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PHARMACOLOGICAL STUDIES ON HEMLOCK WATER DROPWORT

BY

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From the Department of Pharmacology, University of Cambridge

(RECEIVED OCTOBER 24, 1955)

The Umbellifer, *Oenanthe crocata*, is found in damp situations in many parts of the British Isles and Western Europe, and is held to be one of our most poisonous indigenous plants. Confusion in nomenclature renders many early reports unreliable, though Vaughan (1722) appears to have recognized the effects produced in man by ingestion of the tubers. Bloc (1873), Demoor (1896, 1897) and Witthaus (1911) report 124, 218, and 159 cases of poisoning respectively, with death rates of between 26 and 44%. In Demoor's series, the mortality among those who suffered convulsions was approximately 70%. Fatal instances of poisoning still appear in the contemporary literature (*Lancet*, 1947).

Clarke, Kidder, and Robertson (1949) were the first to obtain crystalline oenanthotoxin, and their paper should be consulted for references to earlier work. The extensive studies of Anet, Lythgoe, Silk, and Trippett (1953) have revealed much of its chemical nature, and a structural similarity to some antibiotics such as mycomycin and to related substances obtained from certain Basidiomycetes.

Boehm (1876) suggested that the convulsant properties of oenanthotoxin were similar to those of picrotoxin, a view supported by Grollman and Slaughter (1947). We are unaware of any detailed studies of the pharmacological actions of this plant and present our own in the following paper.

METHODS

Preparation of Material

Tubers of *Oenanthe crocata* were gathered from the Botanic Garden, Cambridge, in September, October, and March of the same and different years. They were stripped of stems and rootlets, cut into thin discs, dried to constant weight at 40° C., and then ground to a fine powder. The powders were extracted with 70% alcohol either by cold percolation or by hot continuous extraction (*British Pharmacopoeia*, 1953). The tinctures obtained by the first method were used as such; but, after hot percolation, water soluble crystals were deposited on cooling; these were discarded when it was found that they had no observable effect on intravenous injection into unanaesthetized rabbits. The final volumes of tinctures were such that 1 ml. corresponded to 0.5–1 g. of dried powder, but much variation in potency was expected because of obvious seasonal differences in the appearance of the tubers. The tinctures were stored at 4° C. and some samples of powder at room temperature.

In later work 4.3 kg. of tubers were obtained from South Devon in January and 8.7 kg. from Kent in February. The tubers were minced, placed in small muslin bags and extracted with 5–6 l. of ether in a 20 l. bolt-head flask standing in a large electrically heated water-tank. The ether distilled into a receiver outside the tank until about 1 l. remained in the bolt-head flask. This residual liquor was driven into a 5 l. flask also standing in the tank, whence it distilled into the same receiver as did the contents of the 20 l. flask. The distilled ether was next drawn back into the extraction flask and the procedure repeated as often as required. The marcs were expressed and re-extracted, the process ending when the ether in the extraction flask remained colourless. Further processes were carried out on the concentrate in the 5 l. flask and were essentially those of Anet *et al.* (1953) except that 40% instead of 20% NaOH was used as a precipitant. After the initial crops of crystals had been recovered from the various solvents, further crops were obtained from many of them by the addition of light petroleum. The total yields were 270 and 234 mg./kg. from the Devon and Kent samples respectively. The melting point of the crystals was 86° C.; Anet *et al.* (1953) found m.p. 87° C. The crystals (oenanthotoxin) were sealed in ampoules under nitrogen and kept at −20° C. They were soluble in propylene glycol and some specimens were stored under similar conditions in this solvent.

When freshly prepared crystals of oenanthotoxin were exposed to air they rapidly became brown; but stored at −20° C., in nitrogen, no change was observed after nine months. Under such conditions solutions of oenanthotoxin in propylene glycol showed no appreciable loss of convulsant properties; dried powders still furnished convulsant tinctures after four months at room temperatures. Tinctures were potent after a similar period at 4° C. The subsequent experiments were carried out using test solutions not more than three weeks old.

Animal Experiments

Most of the observations were made upon unanaesthetized rabbits after the intravenous injection of either 0.5–1 ml. oenanthe tincture or 1 mg./kg. oenanthotoxin in propylene glycol. Cats received similar injections. Oenanthotoxin was also administered intraperitoneally to mice and by stomach tube to rabbits and mice. The behaviour of unanaesthetized animals after such treatment was observed and compared with the effects of picrotoxin given intravenously in rabbits and intraperitoneally in mice.

Central Nervous System.—In cats, similar studies were made on hypothalamic, mid-brain and spinal preparations, and in some animals after decapitation and destruction of the spinal cord. Injections were also given after subaxial section of the cord. In rabbits, only mid-brain, spinal, and pithed preparations were used. Blood pressure was recorded in all these experiments. Respiratory movements were recorded in those animals in which artificial respiration was not required.

Respiration and Blood Pressure.—Changes in blood pressure and respiration produced by oenanthotoxin or oenanthe tincture were studied in intact rabbits anaesthetized with paraldehyde, pentobarbitone, or hexobarbitone. In later experiments, in addition to the blood pressure, the heart action in the open chest was recorded by means of a thread passed through the apex and operating a small Starling heart lever. The heart was protected from interfering respiratory movements by a plastic ring inserted within the pericardium and fixed in position near the base of the heart by an encircling ligature. This ring was attached to a slender steel rod which was clamped outside the chest. The effects of bilateral vagotomy and of atropine upon the response of the cardiovascular system to oenanthotoxin were observed in such preparations. In many of these experiments the complicating convulsions were prevented or arrested by the use of tubocurarine. Similar studies were made on decapitate rabbits after destruction of the spinal cord. These animals received 10 ml. of " Dextraven " (6% w/v Dextran in 0.9% saline) after decapitation, and their blood pressures were maintained subsequently by the continuous intravenous injection of 0.12 ml. of saline/min. containing 100 μg. noradrenaline bitartrate (Light and Co.), delivered by a motor-operated syringe.

Heart.—Oenanthotoxin is insoluble in water and satisfactory suspensions could not be obtained using lecithin as suggested by Clarke *et al.* (1949). When oenanthotoxin in a minimal volume of propylene glycol was added to plasma with gentle shaking, a clear solution was produced after an instant's opalescence. This mixture diluted with Dale–Ringer solution did not lose its convulsant properties after passing through fine filter paper. Perfused isolated rabbit hearts (Brodie and Cullis, 1908) ran well on a mixture of 1% of the rabbit's own plasma and 0.01% propylene glycol in Dale–Ringer solution. When constant values for heart rate, excursions and coronary flow had been obtained from such a preparation, the perfusion fluid was changed to a similar one containing the desired concentration of oenanthotoxin.

In the heart-lung preparation in the dog, recordings were made of arterial and venous pressures, and a cardiometer used to indicate the behaviour of the heart. Various amounts of oenanthotoxin in propylene glycol were added to the blood in the perfusion reservoir to give doses corresponding to 0.15–1 mg./kg. in the intact dog. A similar experiment was performed with a tincture which had been evaporated to dryness and re-dissolved in propylene glycol.

Oenanthotoxin-Barbiturate Antagonism

To investigate the antagonism between oenanthotoxin and pentobarbitone each member of two groups of six rabbits received oenanthotoxin or oenanthe tincture by an ear vein. As soon as convulsions were established in the test group they were arrested by the injection of a sufficient volume of a 1% solution of pentobarbitone sodium. The control group received no pentobarbitone. The test group of animals remained under observation for seven days. The relative effectiveness of picrotoxin and oenanthotoxin as stimulants of depressed respiratory activity was compared in rabbits by a method similar to that of Das (1939). Femoral arterial blood pressure records were made in addition to those of respiration. Hexobarbitone sodium was infused into a femoral vein, the correct dose—between 0.7 and 1.4 mg./kg./min.—to maintain a steady depression of respiration being determined by trial. The test substances in propylene glycol or propylene glycol-plasma were introduced into the inferior vena cava through a 1 mm. bore polythene tube passed by way of the other femoral vein. The rate and volume of the injections were the same for both substances. Comparisons started after 1 hour's anaesthesia, the test solutions being injected alternately. After each test the respiratory activity was allowed to return to the initial level before a further injection was given.

Additional comparisons were made in mice. The LD50 for oenanthotoxin was determined in 72 male albino mice maintained on identical diets, and used when between 15–25 g. in groups of similar mean weight. The same intraperitoneal injection technique was always employed, and the external conditions were maintained constant. Oenanthotoxin was dissolved in propylene glycol, suspended in rabbit plasma, and appropriate dilutions made in 0.9% saline. The volume received by a 20 g. mouse was 0.5 ml. containing 0.002 ml. propylene glycol and 0.1 ml. plasma. Other animals received proportionate doses. The LD50 for picrotoxin in saline was similarly determined. After injection, the times of onset of convulsions and death were noted and final mortalities assessed after 24 hr. All dead mice were subjected to autopsy.

Picrotoxin and oenanthotoxin were compared in their capacity to prevent death in mice depressed by large doses of pentobarbitone sodium. A dose-mortality curve for this was determined by intraperitoneal injection into a total of 64 mice. A convenient dose—the LD82—which would kill a high percentage of mice was selected and given to further groups of mice. The first group of

14 mice received no further injections and served as the control, but of the remaining four groups of 15 mice each, two received 1×LD50 and 2×LD50 of picrotoxin respectively and two 1×LD50 and 2×LD50 of oenanthotoxin. These injections were made within 3 min. of the pentobarbitone injections. The mortality after 24 hr. was noted.

In all experiments close attention was paid to the results of control studies with the solvents, since propylene glycol itself, in doses of about 0.5 ml., induces temporary changes in both blood pressure and respiratory activity. The use of plasma allowed the glycol to be diluted, in critical experiments, beyond its active range.

Results

Unanaesthetized Animals.—Convulsions were induced in forty rabbits by the intravenous injection of oenanthotoxin or oenanthe tincture. There was an initial, brief, quiescent period, often absent with doses of 1 mg./kg. of oenanthotoxin, during which the animals showed twitching of the facial muscles and whiskers with increased respiratory rate. There were occasional twitches of the limbs and often marked head retraction with characteristic backward shuffling. Widespread spasms of all four limbs heralded the onset of violent running movements, at the start of which the animal fell upon its side. Usually the " running " occurred in bouts. At the height of these attacks, respiration was impeded and the buccal mucosa was cyanosed. There was some salivation, but micturition and defaecation were not markedly increased. Chewing, and grinding of the teeth were constantly observed. Towards the end, the spasms became less frequent, and after the arrest of respiration the heart was beating feebly. During the action of the drug the pupils were inconstant. Death always occurred within 30 min. after 1 mg./kg. oenanthotoxin, and within 60 min. after 0.5–1 ml. oenanthe tincture.

Six cats, after intravenous injections of oenanthe tincture, behaved similarly except that the limb movements were those associated with scrabbling or scratching rather than running. Bristling of the fur and unsheathing of the claws were usually prominent. Micturition and defaecation were often noted. In two cats death followed within one minute of the injection and there were no convulsions. In three cats given oenanthotoxin the limb movements showed some tonic features and in these animals the increase in respiration was particularly striking.

In 72 mice intraperitoneal injection of oenanthotoxin gave effects comparable to those after intravenous injection to rabbits, though the onset of convulsions was delayed (Table I), and usually preceded by a marked erection of the tail.

The convulsions induced in rabbits and mice were in pattern indistinguishable from those produced by picrotoxin. The oral LD50 for oenanthotoxin in mice was about 7–8 mg./kg., the usual convulsive phenomena being followed by death, but in 6 rabbits doses up to 90 mg./kg. by mouth induced no observable change in behaviour.

Central Nervous System.—In the cat, the usual limb movements were still obtained when all cerebral tissue anterior to the superior colliculus had been removed, but tonic strychnine-like spasms of the limbs with rigid flexion of the trunk were observed in spinal animals, though larger doses were often required to elicit this response. After subaxial section of the spinal cord, clonic jaw and face movements and tonic spasms of the limbs

TABLE I

DOSE-MORTALITY DATA FOR OENANTHOTOXIN AND FOR PICROTOXIN GIVEN INTRAPERITONEALLY TO MICE

The control injection was a 20% solution of plasma in 0.9% saline containing 0.4% propylene glycol, and caused no change in the behaviour of the mice.

Drug	Dose (mg./kg.)	No. of Mice	Av. Wt. (g.)	Animals Convulsing (%)	Av. Time of Onset of Convulsions (min.)	Mortality (%)	Av. Time from Injection to Death (min.)
Oenanthotoxin	2·21	5	17	100	20	100	48
	1·80	10	17	100	24	90	41
	1·47	20	17	75	18	60	50
	1·20	20	16	80	32	50	59
	0·98	10	17	50	36	40	70
	0·80	5	17	40	41	0	—
	0·59	2	17	0	—	0	—
Control	0·5 ml./20 g. mouse	8	18	0	—	0	—
Picrotoxin	12·60	5	16	100	5	100	11
	10·00	10	18	100	7	90	13
	8·00	16	17	94	9	50	17
	6·40	10	17	80	15	40	33
	5·00	10	17	40	18	0	—
	4·00	5	16	20	30	0	—
	3·18	5	17	0	—	0	—

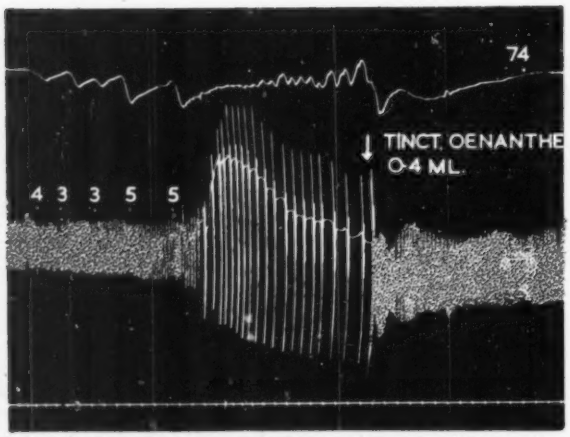

FIG. 1.—Rabbit, 1.2 kg., anaesthetized with pentobarbitone sodium 30 mg./kg. Upper record, arterial blood pressure. Lower record, respiratory movements. Time in 10 sec. Further doses of pentobarbitone (4, 3, 3, 5, and 5 mg.) were given until respiration was greatly slowed. Oenanthe tincture promptly restored the respiratory rate.

followed the intravenous injection of oenanthotoxin. After destruction of the central nervous system no motor activity was seen.

In the rabbit, the mid-brain preparation gave the typical running pattern following administration of the drug, and the other preparations gave results similar to those in the cat. Mid-brain animals showed an increase in respiratory rate and blood pressure. The latter change was absent in the pithed preparation though a slight rise was occasionally seen in the spinal rabbit.

Respiration and Blood Pressure.—In 30 intact rabbits anaesthetized with paraldehyde, urethane or barbiturate there were increases in respiratory rate, or amplitude, or both. These changes were most striking in deeply anaesthetized animals (Fig. 1). The blood pressure after an initial fall—hypotensive phase—usually rose steadily—hypertensive phase—attaining in some cases 160 mm. Hg. Oenanthe tincture and oenanthotoxin gave similar results. The hypotensive phase showed marked variation both in degree and duration in successive similar experiments and was frequently accompanied by a dilatation and slowing of the heart (Fig. 2). Bilateral vagotomy prevented the bradycardia, but the other changes were still seen. During the subsequent hypertensive phase the cardiac dilatation persisted, sometimes accompanied by slowing. These effects were especially marked at the peaks of fluctuations in the blood pressure. This dilatation was increased by bilateral vagotomy which further raised the blood pressure and restored the heart rate. In curarized preparations similar cardiovascular changes were seen. After destruction of the central nervous system in the rabbit, oenanthotoxin gave the usual dilatation of the heart in the hypotensive phase though without cardiac slowing (Fig. 3). The hypertensive phase was absent.

Heart.—Perfusion of the isolated rabbit heart with solutions containing oenanthe tincture or oenanthotoxin caused a reduction of the excursions of the recording lever. The rate was slightly increased with a small decrease in coronary flow. With concentrations in the blood corresponding to 1 mg./kg. body weight in the intact dog, the heart-lung preparation revealed, at constant arterial pressure, an increase in the volume of the heart with a slight rise in venous pressure. When an evaporated tincture was used, these changes were accentuated.

Oenanthotoxin-Barbiturate Antagonism

Sodium pentobarbitone prevented death in six

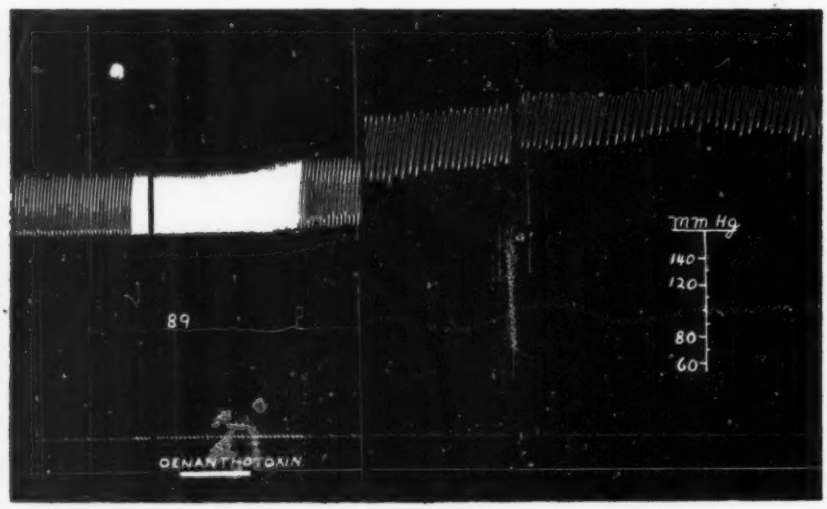

FIG. 2.—Rabbit, 1.8 kg., paraldehyde 1.7 ml./kg. by stomach tube. Heart movements (upper record) recorded in open chest by means of a thread attached to the apex and to a Starling heart lever. Downstroke, systole. Oenanthotoxin i.v. produced progressive dilatation and slowing of the heart. This preparation showed no marked fall in blood pressure (lower record). The hypertensive phase starts towards the end of the tracing. The intervals between the strips are from left to right 39, 78, and 29 sec. Time in sec.

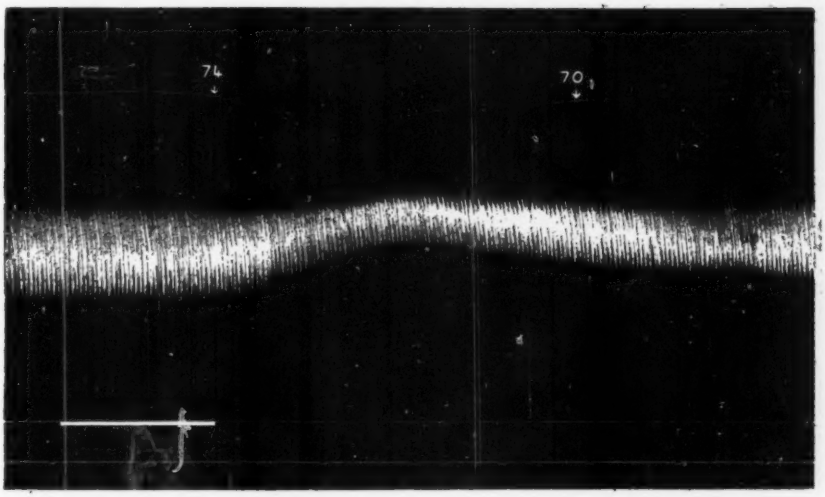

FIG. 3.—Rabbit, 1.9 kg., anaesthetized with pentobarbitone sodium 30 mg./kg., decapitated, and spinal cord destroyed. Blood pressure (upper record) maintained with an infusion of noradrenaline. Oenanthotoxin i.v. at signal produced a fall of blood pressure, impairment of ventricular contraction (lower record), but no cardiac slowing. Time, 0.25 min.

rabbits receiving a lethal dose of oenanthotoxin (Table II), and one of these delivered a living litter on the day after the experiment. In a large series of rabbits, none has survived 0.5–1.0 ml. of oenanthe tincture or 1 mg./kg. of oenanthotoxin without an antidote, but in addition to those shown in Table II many more rabbits and cats thus poisoned have recovered following adequate treatment with pentobarbitone. Fig. 4 shows that when oenanthotoxin was administered to a rabbit under continuous hexobarbitone narcosis a greater and more prolonged increase in respiratory amplitude and rate was observed than that given with picrotoxin in three times the dose. The intraperitoneal LD50 of oenanthotoxin for mice determined by the method of Bliss (1938) was 1.22 ± 0.11 mg./kg., and that of picrotoxin 7.56 ± 1.10 mg./kg. (Table I). The intraperitoneal LD50 of pentobarbitone for mice was 139 ± 1 mg./kg., which agrees closely with the value recorded by Chakravarti (1939). The recovery rates of mice given the LD82 of pentobarbitone were 80% with 1×LD50 of either oenanthotoxin or picrotoxin. With 2×LD50, the recovery rates were 80% and 93% respectively. Deaths in the oenanthotoxin series mainly occurred in the first hour, whereas those after picrotoxin were seen much later in the experiment. Mice receiving oenanthotoxin tended to recover more rapidly from the coma induced by pentobarbitone than those of the picrotoxin series.

TABLE II

OENANTHOTOXIN-PENTOBARBITONE ANTAGONISM IN RABBITS

The dose of oenanthotoxin was 1 mg./kg. i.v., except in experiments 5 and 6 in which 1 and 2 ml. of oenanthe tincture respectively were given i.v. As soon as convulsions were established they were arrested in the test series by the injection of a 1% solution of pentobarbitone. None of the animals given pentobarbitone died.

Expt. No.	Oenanthotoxin Alone (Control)			Oenanthotoxin + Pentobarbitone (Test)		
	Wt. (kg.)	Onset Convulsions after Injection (sec.)	Time after Injection to Death (min.)	Wt. (kg.)	Onset Convulsions (sec.)	Pentobarbitone to control Convulsions (mg.)
1	2·3	65	18·5	2·3	35	40
2	2·2	79	19·7	2·2	50	120
3	1·7	50	19·5	2·4	48	130
4	1·9	77	29·7	1·9	80	110
5	1·2	300	50	1·2	120	60
6	1·4	180	40	1·2	240	90

DISCUSSION

The crystalline product obtained appears similar to the oenanthotoxin of Anet *et al.* (1953) in general chemical and physical properties. The convulsions induced in mice were similar to those described by Clarke *et al.* (1949), but their stated LD50 is lower than ours. Their experimental results, however, are insufficient for an accurate determination of this value. Oenanthe tincture and oenanthotoxin gave qualitatively similar results in all the pharmacological actions examined except that in some cats the oenanthotoxin convulsions tended to be tonic. The similarity between convulsions induced in the mid-brain preparation and the intact rabbit suggests an origin in the brain stem—a view supported by the strychnine-like spasms observed in the spinal animals.

The inconstancy of the cardiovascular response of the rabbit to oenanthotoxin rendered difficult a precise analysis of the mechanisms involved. Since the fall in blood pressure with its associated cardiac dilatation was not prevented by bilateral vagotomy and appeared in rabbits in which the central nervous system had been destroyed, it seemed probable that this was due to a direct action on the heart or an obstruction of pulmonary blood flow. The

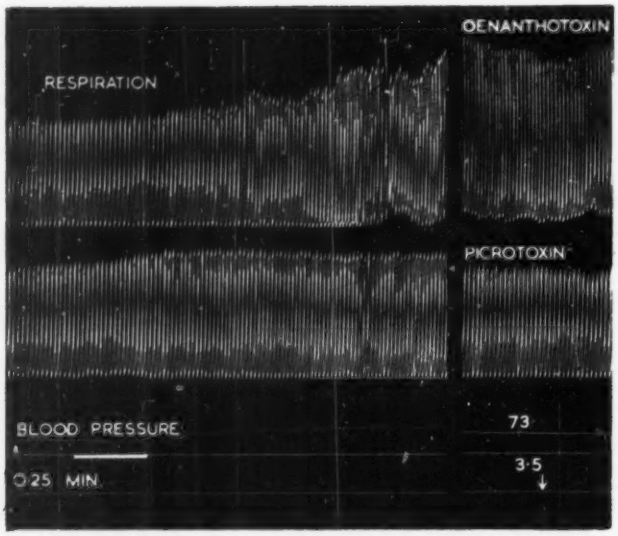

FIG. 4.—Rabbit, 1.8 kg., continuous narcosis with hexobarbitone sodium 1.3 mg./kg./min. Records of respiratory movements. Top line, expt. with oenanthotoxin; 2nd line, expt. with picrotoxin; 3rd line, b.p. in picrotoxin expt. Either 0.2 mg./kg. of oenanthotoxin or 0.6 mg./kg. of picrotoxin i.v. at signal. Arrow in second part of tracing marks 3.5 min. after beginning of each injection. The blood pressure record for oenanthotoxin was similar in form to that for picrotoxin except that the maximum rise was higher by 15 mm. Hg. Oenanthotoxin gave a greater and more prolonged increase in respiratory amplitude and rate than picrotoxin when administered at comparable levels of respiratory depression. Time, 0.25 min.

results obtained with the heart-lung preparation in the dog support the former possibility. The effects of small doses of oenanthotoxin upon the isolated rabbit heart suggest that the major cause of the hypotension is a direct depression of myocardial activity. The slowing of the heart in this phase is probably due to vagal discharge in that it was not seen in pithed rabbits, or after bilateral vagotomy in the intact animal.

Though the hypertensive phase was absent in the pithed animals the blood pressure rise may not be entirely central in origin since these preparations showed a reduced sensitivity to certain peripherally-acting hypertensive agents. Bilateral vagotomy obliterated the bradycardia of the hypertensive phase, increasing the blood pressure and cardiac dilatation. The slowing thus appears to be a reflex response to the raised blood pressure, though a contribution by central stimulation of the vagus is possible. Since the heart dilates as the blood pressure rises and *vice versa*, even in vagotomized animals, it is concluded that the cardiac dilatation in this phase is mainly a mechanical response to the hypertension. Similar responses can also be obtained by the injection of noradrenaline into intact or pithed animals.

Oenanthotoxin has been shown to be more potent than picrotoxin in all the actions examined, but a precise figure cannot be given for their relative effectiveness against pentobarbitone poisoning because of the small number of mice used. The higher early death rate of the mice treated with pentobarbitone and oenanthotoxin may be due to the slow absorption of the latter from the peritoneum.

SUMMARY

1. Crystalline oenanthotoxin and tinctures of *Oenanthe crocata* tubers have similar pharmacological properties.

2. Oenanthotoxin convulsions in rabbits and mice resemble those of picrotoxin; in rabbits and cats they have their origin in the brain stem.

3. Intravenous oenanthotoxin causes a transient fall followed by a sustained rise in blood pressure. The former is due to a direct action on the myocardium.

4. In mice, oenanthotoxin is six times as toxic as picrotoxin, and is more potent as an antidote for pentobarbitone poisoning. In the rabbit, it is a more powerful stimulant of respiration than picrotoxin.

5. Rabbits and cats given a lethal dose of oenanthotoxin can be saved by the early administration of pentobarbitones.

We wish to thank the director of the Botanic Garden, Cambridge, Dr. S. M. Walters, and Mr. P. C. Hall for supplies of *Oenanthe* tubers, and are indebted to Professor E. B. Verney for setting up the heart-lung preparations.

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THE ACTION OF ATROPINE AND HEXAMETHONIUM IN COMBINATION ON GASTRIC SECRETION AND MOTILITY

BY

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The action of atropine is known to vary with the dose, probably owing to a dual action; large doses block the effects of acetylcholine at the periphery, whereas small doses stimulate the medullary centres (Bastedo, 1936; Clark, 1940). We have sought to use the blocking effect of hexamethonium at autonomic ganglia in order to analyse this biphasic response. This study has been made on human subjects using alterations in gastric motility as an index. The second objective was to see if atropine and hexamethonium were additive or potentiating in their acetylcholine-blocking effect, using gastric secretion and motility as indices.

METHODS

In a first series of experiments, five male patients with uncomplicated duodenal ulcer were selected for study of gastric motility, which was recorded by a modification of Carlson's balloon method (Kay, 1947). In each subject, tests were made on each of three consecutive days. The balloon was introduced at 9 a.m. after a 12-hour fast. Control observations were made for 20–40 min. before injecting the test substance. On the first day 0.1 mg. atropine sulphate was given intravenously; on the second day 0.65 mg. atropine sulphate was given intravenously; on the third day 50 mg. hexamethonium iodide was given by intramuscular injection 30 min. before an intravenous injection of 0.1 mg. atropine sulphate.

In a second series of experiments, the effects of atropine and hexamethonium, independently and in combination, were examined on acid secretion and on gastric motility. An object of the experiments was to determine whether the known blocking effects of atropine and hexamethonium are additive or potentiating. A simple way of determining how the interaction of the drugs can be classified (Gaddum, 1953) is to administer half the dose of one necessary for a given effect, with half the corresponding dose of the other. If the combination then causes the effect, the two drugs are additive; if more than the effect there is potentiation. We have modified this method to study the combined action of hexamethonium and atropine on gastric secretion and motility. The effects measured were the development of achlorhydria and inhibition of gastric motility. A combination of the two substances was made: with hexamethonium the effective dosage producing inhibition of acid gastric secretory activity was halved, but with atropine, with which effective dosage is limited by side effects, the standard therapeutic dose was halved. The drugs were given by intramuscular injection, hexamethonium iodide in a dose of 100 mg., atropine sulphate in a dose of 0.65 mg., and for the combined injection 50 mg. hexamethonium iodide with 0.325 mg. atropine sulphate. As there is no chemical incompatibility, the atropine was dissolved in the solution of hexamethonium iodide and the mixture given as a single injection.

Spontaneous secretion of hydrochloric acid, spontaneous night secretion and gastric motility were in turn examined after injection of the drugs alone and in combination.

Spontaneous Secretion of Hydrochloric Acid.—Tests were made on ten patients. A secretion test was given on each of five consecutive days. Control observations were made on the first and last day; the effects of hexamethonium, atropine, and the combined drugs were studied on the intervening days.

Each secretion test was begun at 9 a.m. after a 12-hour fast. The fasting juice was aspirated and the specimens removed every 15 min. thereafter until the end of the series of observations. The control observations were continued for 3 hr. On each of the test days the selected injection was given immediately after the aspiration of the second 15-minute specimen. Special precautions were taken to empty the stomach at each aspiration and all saliva was expectorated. The volume and bile staining of each specimen was noted and the titratable free acid estimated in ml. 0.1N-HCl. Acid output was calculated in milliequivalents.

Spontaneous Night Secretion.—A comparative study was made of the action of hexamethonium, atropine, and hexamethonium with atropine upon the nocturnal secretion of five subjects. The patients fasted from 5 p.m., and a wide bore stomach tube, with multiple perforations at its tip, was passed at 8 p.m. Continuous gastric aspiration was performed using the constant suction provided by an electric pump until 8 a.m. Sedatives were not given. Tests were made on four successive nights. The first night provided control observations and no injections were given. On subsequent nights the drug or combination of drugs to be

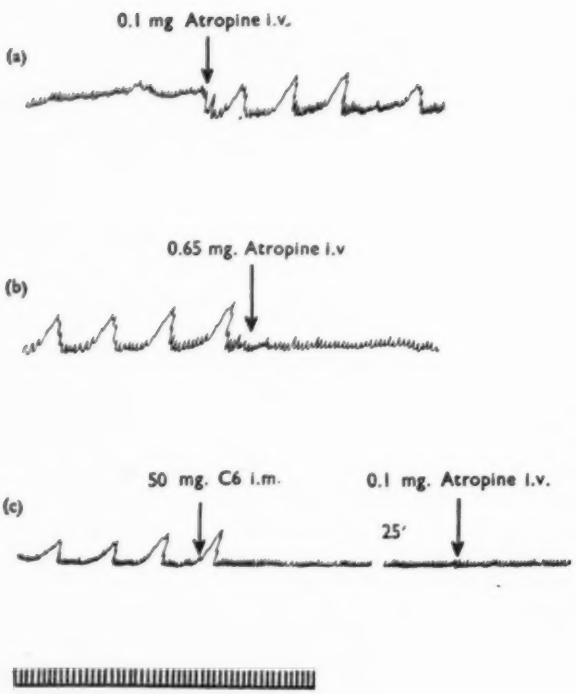


FIG. 1.—Tracings of gastric motility showing (a) the *stimulant* action of 0.1 mg. atropine sulphate i.v., (b) the *inhibitory* action of 0.65 mg. atropine sulphate i.v., and (c) prevention of the stimulant effect of atropine (2nd arrow) by previous administration of 50 mg. hexamethonium i.m. (1st arrow).

tested was given intramuscularly at 8 p.m., midnight, and 4 a.m.

Gastric Motility.—Motility recordings were taken from the ten patients on whom the studies on spontaneous secretion had been made. On each of three successive days each patient was given hexamethonium, atropine, or the combined injection in the doses already described. The injections were made following control periods of observation varying from 35 to 40 min. during which pronounced gastric contractions were observed. The order in which the three drugs were given was varied in each patient.

RESULTS

In the first series of five male patients with uncomplicated duodenal ulcer it was confirmed that the effect of atropine varies with the dose. The intravenous injection of 0.1 mg. atropine, purposely given during a phase of relative quiescence, produced an increase in gastric contractions for 5 to 10 min. in four patients and no change in the existing high amplitude contractions in the remaining patient. On the contrary, the intravenous injection of 0.65 mg. atropine, purposely given during a phase of activity, produced immediate and complete inhibition of the gastric movements in all patients. The intramuscular administration of 50 mg. hexamethonium, as has been reported previously (Kay and Smith, 1950), resulted in complete inhibition of gastric motility after a brief latent period. This inhibitory phase was well established when 0.1 mg. atropine was given intravenously. In each of the five tests atropine failed to stimulate gastric movements (Fig. 1).

In view of this result—namely, that atropine has both central-stimulant and peripheral-inhibitory effects—it was thought that hexamethonium combined with atropine might reduce the acidity of the gastric juice and the motor activity of the stomach. The effects of atropine at the parasympathetic nerve endings might be expected to enhance the block produced by hexamethonium at the ganglia.

The results are now recorded of injecting these substances alone or in combination, on spontaneous gastric secretion of hydrochloric acid, spontaneous night secretion, and gastric motility. Side effects have been noted throughout.

The effect of the combined injection was firstly assessed on the spontaneous secretion of hydrochloric acid.

In 9 of the 10 subjects, the combined injection produced a dramatic fall in the level of free acid with a period of complete achlorhydria of from 1 to 5 hr. The achlorhydric phase was the more prolonged in 8 cases after the combined injection (Table I). The specimens with no free acid were further characterized by low volume and absence of bile. In the remaining patient the titratable acid was reduced from 105 to below 30 ml. 0.1N-HCl. Achlorhydria followed the single injection of atropine in three subjects, but it was not maintained for more than 1 hr.; the volume of the

TABLE I

COMPARISON OF HEXAMETHONIUM, ATROPINE, AND HEXAMETHONIUM+ATROPINE ON INHIBITION OF SPONTANEOUS SECRETION OF HYDROCHLORIC ACID

Patient No.	Hexamethonium (100 mg.)		Atropine Sulphate (0·65 mg.)		Hexamethonium (50 mg.)+Atropine (0·325 mg.)	
	Control Acid Output (mEq./hr.)	Duration of Anacidity (hr.)	Control Acid Output (mEq./hr.)	Duration of Anacidity (hr.)	Control Acid Output (mEq./hr.)	Duration of Anacidity (hr.)
1	3·64	4	3·60	0	4·80	4¾
2	7·28	4	4·56	1	7·84	4¾
3	6·48	1¾	6·24	0	6·52	4
4	3·44	3	3·00	0	3·76	3
5	5·12	1	4·16	1	4·32	4½
6	3·80	2	3·68	0	3·60	5¼
7	3·52	1¾	2·48	0	2·32	3½
8	7·20	3½	8·48	¼	8·80	5
9	16·38	0	16·08	0	16·84	0
10	3·68	1½	4·56	0	5·24	2

TABLE II

COMPARISON OF HEXAMETHONIUM, ATROPINE, AND HEXAMETHONIUM+ATROPINE ON NIGHT SECRETION

Drugs given at 8 p.m., midnight, and 4 a.m.

Patient No.	Control			Hexamethonium (100 mg.)			Atropine (0·65 mg.)			Hexamethonium (50 mg.) +Atropine (0·325 mg.)		
	Volume (ml.)	Free HCl (Clinical Units)	mEq./ hr.	Volume (ml.)	Free HCl (Clinical Units)	mEq./ hr.	Volume (ml.)	Free HCl (Clinical Units)	mEq./ hr.	Volume (ml.)	Free HCl (Clinical Units)	mEq./ hr.
1	556	87	4·03	150	36	0·45	134	56	0·63	70	33	0·19
2	1,460	72	8·76	715	22	1·31	600	55	2·75	450	20	0·75
3	850	60	4·25	325	14	0·39	350	48	1·40	300	10	0·25
4	1,015	64	5·41	370	12	0·37	320	50	1·33	210	12	0·21
5	520	25	1·08	180	5	0·08	210	18	0·32	165	5	0·07

specimens was diminished in a further four cases.

Secondly, the effect of the combined injection was assessed on the spontaneous night secretion.

All three drugs reduced the volume of the night secretion by more than half. The acidity of the gastric juice was markedly reduced by hexamethonium and by the combined injections, but reduced to a slight extent only by atropine alone. In these five experiments, the combined injection was at least as active, and sometimes more active, than the effect of hexamethonium alone (Table II).

Motility recordings were taken from the 10 patients on whom the studies on spontaneous secretion had been made. They again showed the enhanced action of the combined injection. In each case, after a brief latent period, gastric motility was completely inhibited by 100 mg. hexamethonium and by 50 mg. hexamethonium plus 0.325 mg. atropine. It will be seen that the combined injection was at least as effective on 8 subjects in diminishing gastric contractions as hexamethonium alone (Table III). The duration of inhibition after the combined injection varied from 2½ to 4 hr. It is of interest that 0.65 mg. atropine failed to inhibit motility in 5 subjects

TABLE III

COMPARISON OF HEXAMETHONIUM, ATROPINE, AND HEXAMETHONIUM+ATROPINE ON INHIBITION OF GASTRIC MOTILITY

Patient No.	Duration of Inhibition of Gastric Motility (min.)		
	After Hexamethonium (100 mg.)	After Atropine (0·65 mg.)	After Hexamethonium (50 mg.)+ Atropine (0·325 mg.)
1	170	90	180
2	80	0	180
3	150	120	190
4	180	0	150
5	105	0	160
6	195	0	240
7	125	70	185
8	110	50	175
9	150	0	150
10	180	80	220

although this dose, when given intravenously, is invariably effective.

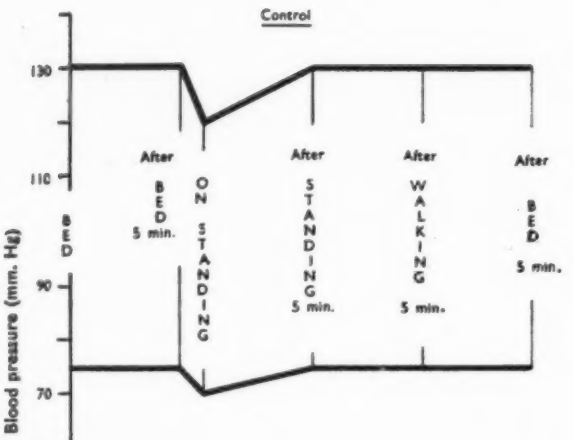
Side Effects.—In the foregoing studies on spontaneous secretion and gastric motility, 100 mg. hexamethonium, 0.65 mg. atropine, and the combination of drugs, 50 mg. hexamethonium plus 0.325 mg. atropine, have each been administered on two occasions to each of 10 subjects. The frequency and severity of the known side-effects of the drugs have been noted.

Impairment of accommodation with resultant blurring of vision was experienced by 9 patients after hexamethonium, by 5 after atropine, and by 4 after the combined injection. Dryness of the mouth was volunteered by 2 patients after hexamethonium, by 8 after atropine, and by 1 after the combined injection.

As these injections were given to recumbent subjects it is not surprising that none complained of faintness or lightheadedness.

The hypotensive effect of the drugs under investigation has been studied in greater detail in five males aged 22, 28, 30, 45 and 52 years. Before giving the drug to be tested, blood-pressure readings were made with the subject at rest in bed, immediately on standing, after standing erect for 5 min., after walking for 5 min., and finally, after being recumbent for 5 min. Similar observations were made 1 hr. after an intramuscular injection of 100 mg. hexamethonium, after 0.65 mg. atropine, and after 50 mg. hexamethonium plus 0.325 mg. atropine. The three tests were made on separate days.

In the control observations, and after atropine, a slight fall of blood pressure occurred immediately on standing (5–10 mm. Hg). This fall was transient and was quickly overcome by compensatory vasoconstriction. After hexamethonium the resting blood pressure was reduced and the fall of pressure on standing was considerable and persisted while the subject was erect (45, 20, 15, and 30 mm. Hg). The fifth patient had a syncopal



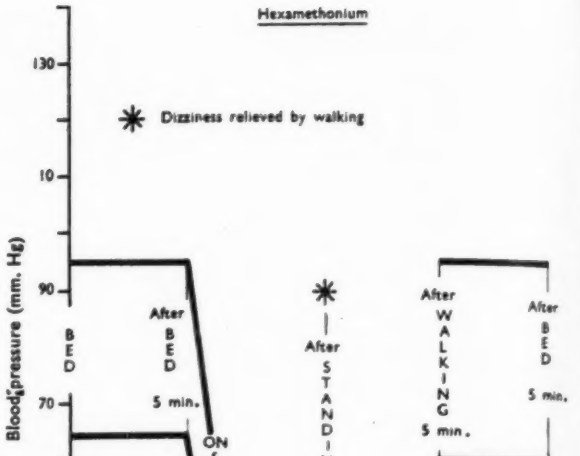
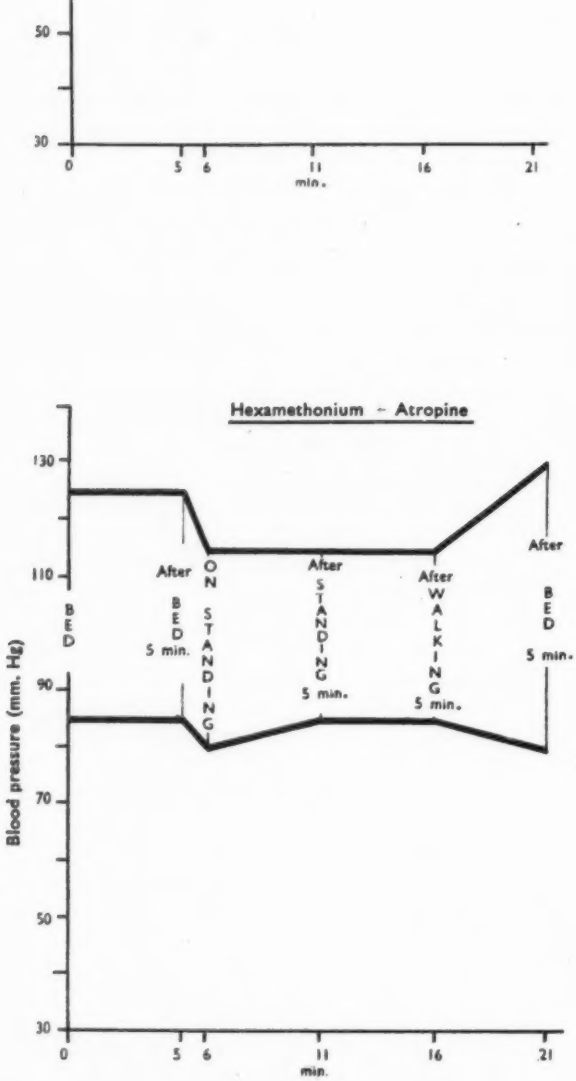


FIG. 2.—Blood pressure changes in a man aged 52 years, (a) at rest in bed; (b) immediately on standing; (c) after standing erect for 5 min.; (d) after walking for 5 min.; (e) after being recumbent for 5 min.



attack and a blood pressure reading was not obtained for the standing position. After hexamethonium plus atropine, the resting blood pressure was only slightly lower than the control reading and the fall of pressure on standing, though persisting while the subject was erect, was less than after hexamethonium alone. The findings in the man aged 52 are shown in Fig. 2.

It will be noted that bodily activity causes a compensatory rise in blood pressure when it has fallen after hexamethonium. The severity of blood-pressure changes are in general more marked with hexamethonium alone than when the smaller dose is given in combination with atropine.

DISCUSSION

The most important action of atropine is on the effector cells in the distribution of the cholinergic fibres of the autonomic system, rendering them insensitive to acetylcholine. Meyers and Abreu (1952), however, found that the effects of atropine vary with the dose; either lethargy or excitement, for instance, may occur under special conditions in the human subject. They observed temporary

bradycardia which they attributed to preliminary medullary stimulation, but this feature was later obscured by the more usual tachycardia associated with the predominant action of the drug. The biphasic response has been further examined by Anderson (1952), who found initial slowing of the pulse followed by an increase, and, in studies on gastric tone, an increase followed by a decrease.

This two-fold action of atropine has been analysed by using the specific ganglion-blocking effect of hexamethonium. The stimulant effect of atropine is not seen when the parasympathetic ganglia have been paralysed, but the possibility exists that this action is exerted at the ganglia. This is, however, unlikely, for pulse rate and mental function are also influenced by particular doses of atropine. Thus the stimulation is probably central.

Hexamethonium, atropine and hexamethonium combined, and atropine alone, have been compared for their actions on gastric secretion and motility. We have confirmed that atropine in a dose of 0.65 mg. intramuscularly is not a reliable inhibitor of gastric secretion and motility. Its main effect on secretion is to diminish the volume of the gastric juice without altering appreciably the acid concentration. In this group of subjects hexamethonium combined with atropine had a marked effect on gastric secretion—usually greater than the effect of hexamethonium alone. Furthermore, the combination of the two drugs, each in half the dose given alone, was relatively free from side effects; in particular the postural hypotension of hexamethonium was greatly reduced. The effectiveness of the two drugs in combination may be due to a complex of actions. Ganglion blockade by hexamethonium blocks the transmission of central stimuli due to atropine. The preganglionic stimulation produced by atropine may sensitize the ganglion to hexamethonium and so augment its effect (Paton, 1951). Ganglion blockade may depress the peripheral production of acetylcholine and enhance the inhibitory action of atropine on the tissues (Grossman and Robertson, 1952).

We have confirmed that, whereas large doses of atropine inhibit gastric motility, small doses stimulate it; preliminary blockade of the parasympathetic ganglia by means of hexamethonium prevents this stimulant action of atropine. While it can thus be shown that both drugs have properties which lead us to expect that they will reduce the motor and secretory activity of the stomach, it is unlikely that this combination of the two will have general clinical value. Though the use of halved doses has at least an additive, and even a potentiating effect, and reduces the side effects, it is unlikely to have a practical application, for the combination of drugs must be given by injection and such a method is inapplicable for the treatment of peptic ulcer, where a large number of patients require treatment over long periods.

Summary

1. It is confirmed that, in human subjects, atropine has both central stimulant and peripheral inhibitory effects, and that hexamethonium is capable of blocking its central action.

2. The effect of hexamethonium alone and of atropine alone on gastric secretion is shown, in general, to be less than the effect of the drugs given in combination in half the dose.

3. Similar results were obtained in studies of gastric motility.

4. The drugs in combination in half-doses produce fewer side effects than do hexamethonium alone or atropine alone.

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EFFECT OF ANAESTHETICS AND HAEMORRHAGE ON THE RELEASE OF NEUROHYPOPHYSIAL ANTIDIURETIC HORMONE

BY

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The first demonstration of "physiological" or "pharmacological" activity in extracts from the posterior pituitary gland was made by Oliver and Schäfer (1895) when they discovered that these extracts raise the blood pressure. Since then, although the role of neurohypophysial principles in the physiological control of the renal excretion of water and of milk ejection has been well established, very little evidence has been adduced which indicates a physiological role for the pressor action of neurohypophysial antidiuretic hormone.

Rydin and Verney (1938) showed that removal of blood from arteries in dogs resulted in an antidiuretic response which they concluded "was humorally determined and that the humoral agent was not adrenaline." Ginsburg and Heller (1953b) found that the antidiuretic activity in blood increased during haemorrhage in anaesthetized rats, and they concluded that there was increased release of antidiuretic hormone from the neurohypophysis. The amounts of antidiuretic hormone found in blood were large enough to suggest that it may have had an effect on blood pressure in these conditions. The effect of haemorrhage on the neurohypophysis has therefore been more fully investigated, with particular reference to the anaesthetics used.

METHODS

Young adult rats (170–270 g.) of the albino Wistar strain were anaesthetized with ether, pentobarbitone sodium (65 mg./kg. intraperitoneally), urethane (2.0 g./kg. subcutaneously), chloralose (60–100 mg./kg. intravenously), or ethanol (3–5 ml./kg. intravenously).

Ethanol (30% v/v in 0.9% NaCl solution) and chloralose were injected into conscious animals through a polyethylene catheter which had been inserted in a femoral vein under ether anaesthesia 18 hr. previously.

The trachea, femoral vein, and the cephalic end of an external jugular vein were cannulated. Blood pressure was recorded from a femoral artery, rather than a carotid artery, in order to avoid disturbance of cerebral circulation.

"Slow" Haemorrhage.—In most experiments 0.5 ml. blood/100 g. body wt. was withdrawn from the external jugular vein at 4 min. intervals until 5–7 such withdrawals had been made. When blood samples (0.2–0.9 ml.) for biological assay were taken, an equal volume of rat blood was simultaneously returned into the animal through the femoral vein. The first samples for assay were taken two minutes before the first haemorrhage, and subsequent samples were taken in the middle of the interval between haemorrhages.

"Rapid" Haemorrhage.—In a few experiments the net loss of blood was achieved more quickly and remained more or less constant during the collection of the samples for assay. The barrel of a 10 ml. syringe was attached by polyethylene tubing to a cannula in a femoral artery. The syringe was held lower than the animal and blood was allowed to flow into it. When 5–6 ml. of blood had been withdrawn the syringe was raised so that the level of blood in it was 40–45 cm. above the rat's heart. In this way the blood pressure of the rat was maintained at 32–36 mm. Hg. Samples of blood for assay were withdrawn from an external jugular vein without net blood loss, as described above.

Both vagi were cut in the neck about 15 min. before blood withdrawal in some experiments. In others both carotid sinus areas were denervated. Since, after acute carotid sinus denervation, rats do not tolerate haemorrhage satisfactorily, this operation was performed under ether anaesthesia 18 hr. previously.

Extraction of Neurohypophysis.—Animals from which the neurohypophysis was removed for assay were killed by decapitation. In unanaesthetized rats this was done without excitement or even the need to hold them firmly. They were introduced into a cardboard tube open at both ends, about 2½ in. in diameter and 10 in. long. As the rat's head emerged from the opposite end of the tube it was decapitated by a single blow from an axe. The pituitary was dissected out at once and the neurohypophysis was separated from the adenohypophysis. After weighing, the neurohypophysis was homogenized in 0.5 ml. of 0.25% (w/v) acetic acid in a Potter–Elvejhem ground glass homogenizer. The homo-

genate and washings (3 times with 0.5 ml. of 0.25% acetic acid) were placed in a boiling water-bath for 5 min. and, after cooling, were filtered. The pressor and oxytocic potencies of the extracts were usually assayed on the same day as the preparation of the extract and on the day following. The extracts were stored in a deep-freeze cabinet at −20° C.

Assay Methods.—The method of Ginsburg and Heller (1953a) was used for the assay of antidiuretic activity by intravenous injection into unanaesthetized, water-loaded rats, with the modifications that the bladders of the animals were cannulated and that the urine volume was measured at 5 min. intervals. With blood samples of low antidiuretic activity, a (2+1) assay design was used. Otherwise a (2+2) procedure was adopted.

Oxytocic activity was assayed on the isolated virgin rat uterus as described by Holton (1948). The uterus was suspended in a 30 ml. bath in a modified Locke's solution at 30° C., and unknowns and standards were added at 3 min. intervals.

Dekanski's (1952) method was used for the assay of vasopressin on the rat's blood pressure. Rats weighing 230–300 g. were anaesthetized with urethane (0.8 ml./100 g. of a 25% w/v solution, subcutaneously). Blood pressure was recorded from a carotid artery and injections were given into a femoral vein. The rats were given dibenamine (10 mg./kg.) intravenously in three divided doses.

Pitressin (Parke Davis & Co.) was used for standards in pressor and antidiuretic assays and Pitocin (Parke Davis & Co.) was used in oxytocic assays.

Results

Antidiuretic Activity of Blood During Gradual Haemorrhage.—Figs. 1 and 2 show the changes in the antidiuretic activity of external jugular blood during the loss of successive aliquots of 0.5 ml. of blood/100 g. body weight at 4 min. intervals, in rats anaesthetized with ether, pentobarbitone, ethanol, urethane, and chloralose. Increases of more than 100-fold in the antidiuretic activity of the blood were found, and in order to illustrate such large changes on a single figure the antidiuretic activity of the blood has been plotted on a logarithmic scale.

The results of representative experiments with ether, pentobarbitone, and urethane are shown on Figs. 1a, 1b, and 1c respectively. Qualitatively and quantitatively, the results obtained are similar and they will therefore be discussed jointly. The samples of external jugular blood taken before haemorrhage had antidiuretic activity which was equivalent to 0.1–0.25 mU. vasopressin/ml. When five aliquots had been withdrawn from the rats, the antidiuretic activity of the blood was approximately one hundred times greater than that in the initial samples, and was in the range of 7.0 to 25 mU. vasopressin/ml. blood.

In some experiments the blood pressure did not fall by more than 30 mm. Hg until three aliquots had been removed (Fig. 1a); in other experiments the blood pressure fell by 40 mm. Hg or more after the withdrawal of only one aliquot (Figs. 1b and 1c). The first significant rise in antidiuretic activity of external jugular blood seemed to be associated with the first pronounced fall in blood pressure. For example, in a rat (Fig. 1a) anaesthetized with ether, after the removal of the first two aliquots, the blood pressure fell from 108 to 80 mm. Hg and the antidiuretic activity of the blood only changed from 0.12 to 0.18 mU. vasopressin/ml. After the withdrawal of the third aliquot the blood pressure fell to 35 mm. Hg and the antidiuretic

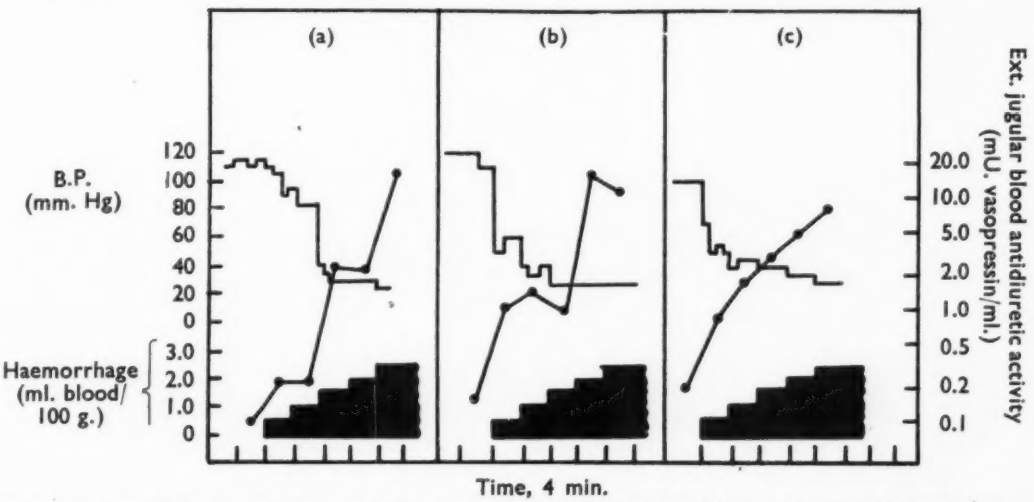

FIG. 1.—Effect of slow haemorrhage upon blood pressure, and antidiuretic activity in external jugular blood, in rats anaesthetized with (a) ether, (b) pentobarbitone, and (c) urethane. 0.5 ml. blood/100 g. was withdrawn at 4 min. intervals. ——— blood pressure, mm. Hg; •———• mU. vasopressin/ml. blood.

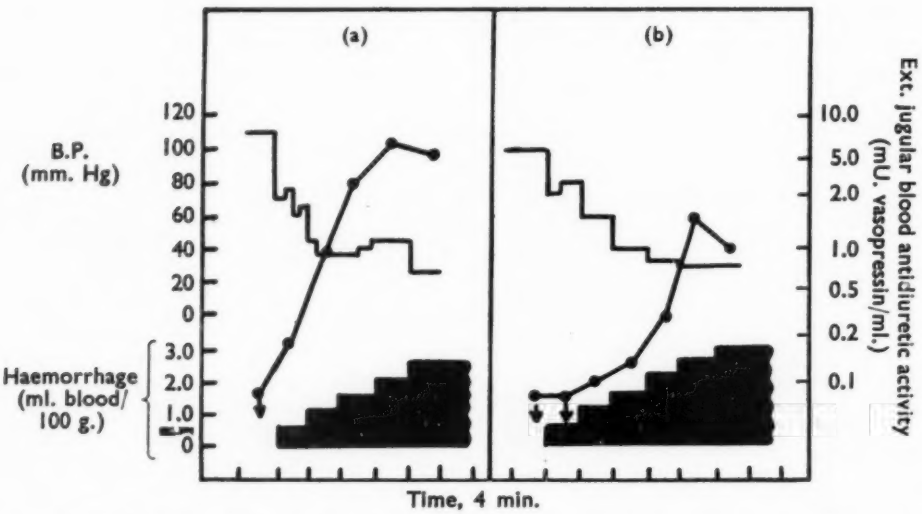

FIG. 2.—Effect of slow haemorrhage upon blood pressure, and antidiuretic activity in external jugular blood, in rats anaesthetized with (a) ethanol and (b) chloralose. 0.5 ml. blood/100 g. was withdrawn at 4 min. intervals. ——— blood pressure, mm. Hg; •———• mU. vasopressin/ml. blood.

activity of the blood increased to 2.0 mU./ml.; the antidiuretic activity was thus more than ten times greater than that of the previous sample and nearly 20 times more than in the initial sample of external jugular blood. In a rat (Fig. 1b), anaesthetized with pentobarbitone, after the removal of the first aliquot the blood pressure fell from 110 to 50 mm. Hg and the antidiuretic activity of the external jugular blood increased from 0.18 to 1.0 mU./ml.

The antidiuretic activity in the blood increased further as more blood was removed. In some experiments it rose gradually with each successive loss of blood. In other animals the antidiuretic potency remained for a while unchanged (within the probable limits of error of the assays), during the withdrawal of two or three samples, and then there was a second sudden and marked increase to levels between 15 and 25 mU./ml. With each of the three anaesthetics results of both types were obtained.

Three experiments were performed on rats anaesthetized with ethanol. Fig. 2a shows the results of a typical experiment. In the first sample

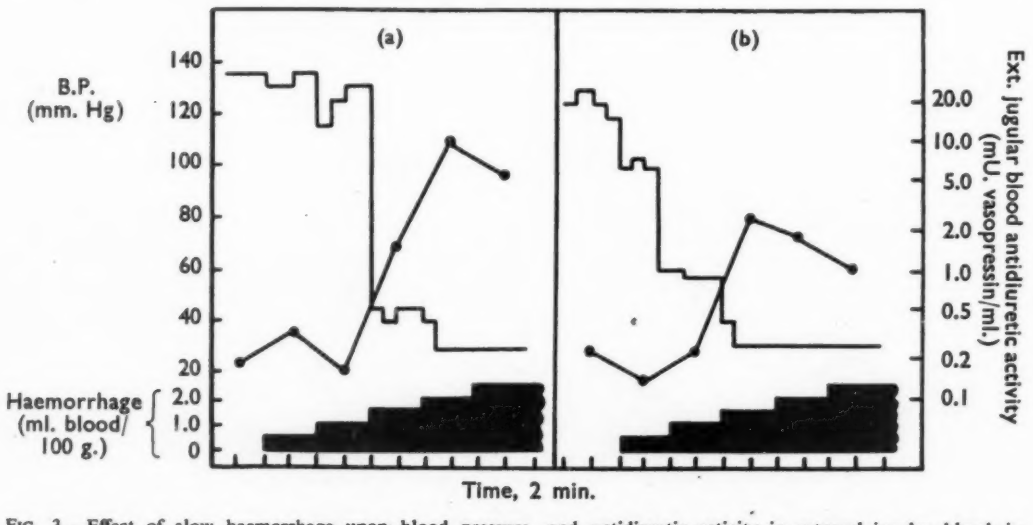

FIG. 3.—Effect of slow haemorrhage upon blood pressure, and antidiuretic activity in external jugular blood, in rats anaesthetized with pentobarbitone (a) after bilateral vagotomy and (b) after denervation of carotid sinuses and bilateral vagotomy. ——— blood pressure, mm. Hg; •———• mU. vasopressin/ml. blood.

of external jugular blood tested, before haemorrhage, no antidiuretic activity could be detected (i.e., less than 0.05 mU./ml.). When the blood pressure fell by 40 mm. Hg or more, the antidiuretic potency of the external jugular blood increased and was detectable. It continued to increase as more blood was removed, but without any obvious irregularities such as those shown on Figs. 1a and 1b. The greatest antidiuretic activities which were found in external jugular blood after the removal of five aliquots were of the order of 5 mU./ml. of blood, which is rather less than in comparable blood taken from rats anaesthetized with ether, pentobarbitone, or urethane.

In animals anaesthetized with chloralose, the antidiuretic activity of the blood was much less than when other anaesthetics were used. Even after the removal of five aliquots of blood, in two out of three animals, the antidiuretic activity of the blood was too low to be detected, i.e., <0.2 mU./ml. blood. In the third rat (see Fig. 2b) the highest concentration was 1.6 mU./ml., which is 3–4 times less than that observed with ethanol and 5–15 times less than that in rats anaesthetized with ether, pentobarbitone, or urethane.

The blood-pressure changes in rats anaesthetized with chloralose appeared to differ from those with other anaesthetics. The blood pressure fell in regular steps with each withdrawal of blood from 100 mm. Hg or more to about 35 mm. Hg after five or six aliquots had been removed, and a sudden collapse in blood pressure was not noted.

Effect of Bilateral Vagotomy and Denervation of the Carotid Sinuses.—Fig. 3a shows the results of an experiment on a rat under pentobarbitone anaesthesia after acute bilateral vagotomy. Although the initial blood pressure was higher than in rats with intact vagi, the changes in antidiuretic activity in external jugular blood during haemorrhage were essentially the same and are related to the changes in blood pressure. The greatest antidiuretic activity observed was 9.2 mU./ml. blood; in another similar experiment it was 6 mU./ml.

Fig. 3b shows the results of an experiment on a rat under pentobarbitone anaesthesia after denervation of both carotid sinuses 18 hr. previously and section of both vagi 15 min. before the first haemorrhage. The blood pressure fell with each haemorrhage, and the antidiuretic activity of the external jugular blood did not increase until the blood pressure reached 30–35 mm. Hg. The maximum activity was 3.3 mU./ml. blood in the experiment shown on Fig. 3b and less than 5.5 mU./ml. in another similar experiment; in rats under pentobarbitone anaesthesia, with vagi and the innervation of the carotid sinuses intact, the maximum activities were 15.0, 16.8, and 11.0 mU./ml. blood.

Effect of Neurohypophysectomy.—Blood was withdrawn from three rats under pentobarbitone anaesthesia which had been neurohypophysectomized 5–7 days previously. No antidiuretic activity (i.e., <0.1 mU./ml.) was detected in any samples of blood taken from the external jugular vein even after the loss of 6 aliquots of blood. The ability of these rats to maintain a high blood pressure in spite of the removal of up to 2 aliquots did not seem to be less than that of animals with the neurohypophysis intact. However, in the neurohypophysectomized rats the blood pressure fell to less than 20 mm. Hg when 4–5 aliquots had been removed, whereas in rats with neurohypophyses intact the blood pressure was usually above 30 mm. Hg.

Antidiuretic Activity of Blood after "Rapid" Haemorrhage.—The anaesthetic used in all experiments in which blood was removed rapidly from the animals was pentobarbitone. Fig. 4 shows the results of a typical experiment. Over a period of 2 min., 5.5 ml. of blood (approx. 2.5 ml./100 g. body weight) was withdrawn from a cannulated femoral artery. The cannula was attached to a reservoir of blood held with the blood level 44.5 cm. above the rat's heart, and during the subsequent 23 min., 0.75 ml. passed out of the animal into the reservoir. The changes in the antidiuretic activity of the external jugular blood showed two distinct phases. The first sample of external jugular blood, taken 2 min. after the blood pressure had been lowered, had an antidiuretic activity of 4.8 mU./ml. compared with 0.25 mU./ml. in the control sample, taken before any blood loss. The antidiuretic activity of the blood then fell and 12 min. later was only 0.5 mU./ml. The next two samples of external jugular blood showed another rise in antidiuretic potency, and the last sample tested (taken 23 min. after the blood pressure had been lowered) contained 3.0 mU./ml. In two other experiments, similar results showing two apparently separate increases in antidiuretic activity of external jugular blood were obtained.

Effect of Anaesthetics and of Haemorrhage on the Pressor and Oxytocic Activities of the Neurohypophysis

In view of the very high levels of antidiuretic activity found in blood during haemorrhage it seemed possible that the amounts of hormone liberated might cause significant changes in the

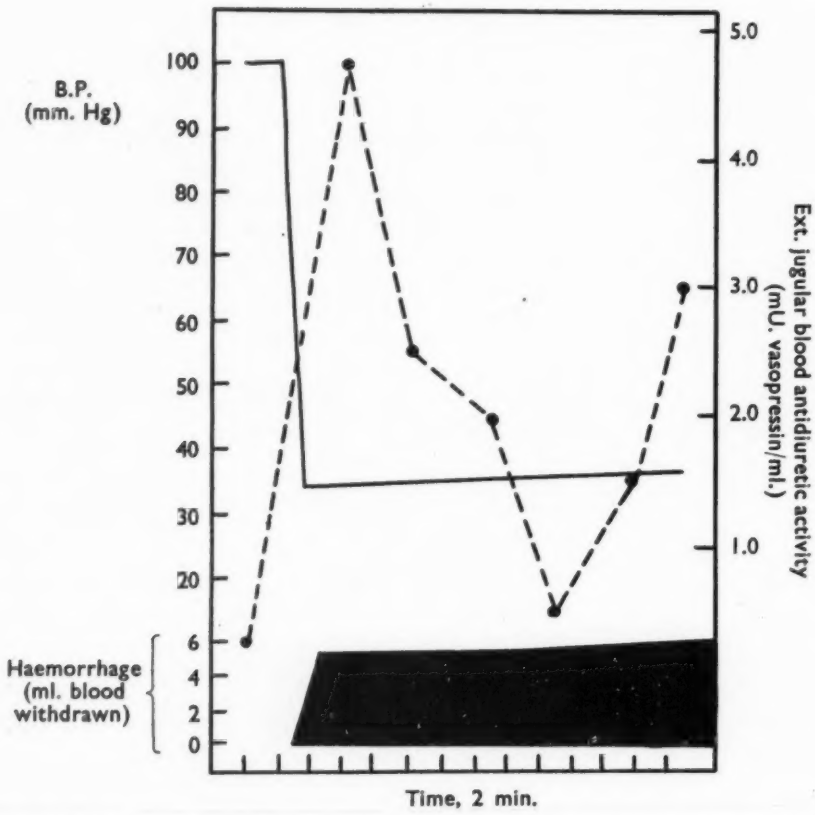

FIG. 4.—Effect of rapid haemorrhage on blood pressure, and antidiuretic activity in external jugular blood. 5.5 ml. blood was withdrawn in 2 min. and the blood pressure was maintained at 33–37 mm. Hg. ——— blood pressure, mm. Hg; •——• mU. vasopressin/ml. blood.

active contents of the neurohypophysis. Table I shows the oxytocic and pressor activities of rat posterior pituitaries obtained from decapitated unanaesthetized rats and rats anaesthetized with pentobarbitone, chloralose, urethane, ether, or ethanol. Half the anaesthetized rats were bled under conditions similar to those of the "slow" haemorrhage experiments (7 haemorrhages, each of 0.5 ml. blood/100 g. body weight at 4 min. intervals); the remainder were anaesthetized for the same period as the rats which were bled, and the carotid and jugular vessels were exposed in the neck. Each gland was extracted separately, and the extracts (with a few exceptions) were assayed for oxytocic and pressor activity.

All the rats from which glands were taken for assay were male albino rats of the Wistar strain of similar weights and ages, but they were obtained from two sources. The supply of rats from source A (another laboratory) could not be repeated and the remainder came from a dealer (source B). The glands of the unanaesthetized rats from A had significantly greater oxytocic and pressor activities than those from B; the ratio of pressor to oxytocic activity in both groups was not significantly different from 1.0.

The activities of glands from animals under chloralose, ether, or ethanol anaesthesia were not significantly different from those from unanaesthetized rats of the same batch, and there was no significant change in the activities of the glands from rats which had been bled.

Rats from both groups, A and B, were anaesthetized with urethane, and the hormonal activity in the glands of rats in the former group was greater than in the latter, as in the unanaesthetized controls. In all rats anaesthetized with urethane, there was more oxytocic activity in the neurohypophysis relative to the pressor activity than in unanaesthetized rats of the same group. The mean ratio of pressor to oxytocic activity in all unanaesthetized rats was 1.12 ± 0.09 (S.E.; 14 observations) compared with 0.49 ± 0.10 (12) in all rats anaesthetized with urethane. This difference is highly significant ($t=3.25$, $P<0.01$). This change seems to be attributable to an increase in oxytocic activity and a decrease in pressor activity in the glands of rats anaesthetized with urethane, compared to those of unanaesthetized animals. Haemorrhage did not affect the hormonal activities of the neurohypophyses of rats anaesthetized with urethane.

The only rats in which haemorrhage had a significant effect on the activities of the neurohypophysis were those anaesthetized with pentobarbitone. The anaesthetic by itself reduced both the oxytocic and pressor activities to a significant extent ($t=12.0$, $P<0.001$ and $t=2.43$, $P<0.05$ respectively) with a greater loss of oxytocic potency so that the pressor/oxytocic ratio was 2.27 ± 0.37 (5) compared with 1.16 ± 0.25 (4) in the unanaesthetized controls from the same batch. The change of ratio is also significant ($t=4.9$, $P<0.001$). In the glands of rats which had been bled under pentobarbitone anaesthesia the ratio of pressor to oxytocic activity was not different from that in the anaesthe-

TABLE I

EFFECT OF ANAESTHETICS AND OF HAEMORRHAGE ON THE OXYTOCIN AND VASOPRESSIN CONTENTS OF RAT NEUROHYPOPHYSIS

Potencies in mU. ± S.E. P/O = ratio of pressor to oxytocic activity. The numerals in parentheses give the number of glands assayed. A and B refer to two different sources from which rats were obtained.

Anaesthetic		A		B		All Rats
		Oxytocic Potency	Pressor Potency	Oxytocic Potency	Pressor Potency	P/O
None		778 ± 34 (4)	902 ± 74 (4)	436 ± 50 (10)	451 ± 43 (10)	1·12 ± 0·09 (14)
Pentobarbitone sodium	Not bled	284 ± 51 (5)	529 ± 119 (8)	–	–	2·19 ± 0·23 (10)
	Bled	432 ± 19 (5)	901 ± 110 (6)	–	–	
Chloralose	Not bled	734 ± 105 (4)	703 ± 87 (4)	–	–	1·11 ± 0·12 (8)
	Bled	752 ± 82 (4)	841 ± 23 (4)	–	–	
Urethane	Not bled	1,470 (2)	465 (2)	685 ± 40 (4)	366 ± 25 (4)	0 49 ± 0·10 (12)
	Bled	1,009 (2)	502 (2)	607 ± 75 (4)	325 ± 30 (4)	
Ethanol	Not bled	–	–	422 ± 57 (4)	421 ± 45 (4)	1·03 ± 0 11 (8)
	Bled	–	–	415 ± 46 (4)	440 ± 100 (4)	
Ether	Not bled	–	–	419 ± 93 (4)	569 ± 63 (4)	1·38 ± 0·17 (8)
	Bled	–	–	425 ± 40 (4)	499 ± 50 (4)	
None, "sham operated"				439 ± 53 (5)	443 ± 31 (6)	1·06 ± 0·13 (5)

tized controls, but absolute activities in the bled rats were significantly higher. After haemorrhage the oxytocic and pressor activities of the glands were 432 ± 19 mU. and 910 ± 110 mU. respectively, compared with 284 ± 51 mU. of oxytocic activity and 529 ± 119 mU. of pressor activity in the rats which were only anaesthetized. These differences between the glands of bled rats and the anaesthetized controls are significant, $t=2.48$, $P<0.05$, and $t=4.0$, $P<0.01$, for oxytocic and pressor activities respectively.

Cavallero, Dova, and Rossi (1952) found that after "sham-adrenalectomy" in rats the antidiuretic potency of the neurohypophysis was reduced. Since the animals which were anaesthetized with ethanol and chloralose had been provided on the previous day with indwelling venous cannulae for the intravenous administration of the anaesthetic, a further series of controls was necessary. Conscious rats were decapitated 18 hr. after an operation under ether anaesthesia during which cannulae were placed in an external jugular vein and in the bladder. There was no difference between the hormonal activities in the glands of the operated rats and those of unoperated controls.

DISCUSSION

Although great increases in the concentration of antidiuretic hormone in the blood were observed during severe haemorrhage it does not necessarily follow that they were due to increased liberation of the hormone from the gland. During haemorrhage, the resistance of vascular beds alters to different extents so that blood flow in the brain, heart and thoracic muscles is reduced to a smaller extent than in the skin, splanchnic area and limb muscles (Franklin, 1951; Mott, 1953). The external jugular outflow in the rat, although largely cerebral (Greene, 1935), also contains effluent from skin, glands, and muscles, and the reduction of the latter during haemorrhage would increase the proportion of cerebral (and pituitary) outflow. However, it is improbable that the neurohypophysial contribution to external jugular blood flow could be increased 100-fold, and an increase of that order would be needed to explain the rise in the antidiuretic activity of blood by re-distribution of blood flow. Moreover, when rats were bled under chloralose anaesthesia, the increase in antidiuretic activity in blood was very much less than

when other anaesthetics were used, but presumably the changes in vascular resistance during haemorrhage were similar.

Reduction in the rate of disappearance of the hormone from the circulation could increase the concentration in blood. In rats, antidiuretic hormone is removed from the circulation by organs in the splanchnic area and the kidneys (Ginsburg and Heller, 1953b and c), and the persistence of the hormone in the circulation will depend, in the first place, on the proportion of the total blood volume perfusing these organs in unit time. This proportion may not be greatly altered by haemorrhage, for, although the hepatic and renal blood flow may be reduced, the total blood volume is also lowered. In "rapid" haemorrhage experiments, where the blood pressure was kept at approximately 35 mm. Hg, antidiuretic activity in blood increased at first and then disappeared, so that it was only 1/20th of its peak value within 12 min. (see Fig. 4). Thus, decreased clearance of antidiuretic hormone from the blood during haemorrhage does not account for the increased concentration of the hormone in the blood.

It seems reasonable therefore to conclude that, during haemorrhage, neurohypophysial antidiuretic hormone is liberated into the circulation in increased amounts. Two distinct and separate phases in this liberation of the hormone could be discerned. The first phase, which was observed in all experiments (except those on neurohypophysectomized rats and in chloralose anaesthesia) was associated with the first considerable fall in blood pressure. This observation agrees with those of Brun, Knudsen, and Raaschou (1945a and b) and Noble and Taylor (1953), who found that persistent oliguria, and the appearance of an antidiuretic substance in the urine after fainting, was due to the brief circulatory collapse. Also, Andersson and Larson (1954) have suggested that the antidiuresis produced by vomiting can be attributed to a rapid fall in blood pressure causing release of antidiuretic hormone from the neurohypophysis. Stimulation of the central end of a divided vagus nerve causes liberation of posterior pituitary hormones (Chang, Chia, Huang, and Lim, 1939; Andersson, 1951) indicating the existence of vago-pituitary connexions. Brun *et al.* (1945b), Nobel and Taylor (1953) and Andersson and Larson (1954) suggest that, following a fall in blood pressure, afferent impulses by way of such connexions stimulate the pituitary. In the present experiments, bilateral vagotomy did not materially affect the increase in the antidiuretic hormone concentration in blood during haemorrhage. Thus, although they may take part, afferent impulses in the vagus nerves in rats do not play a decisive role in the mediation of this response to a fall in blood pressure. This finding may be peculiar to rats, since Blood, Kosman, and D'Amour (1955) have shown that, although pulmonary depressor reflexes can be elicited in rats, they are not abolished, as in other species, by vagal section. The results obtained after denervation of the carotid sinuses plus bilateral vagotomy are difficult to interpret. The antidiuretic activity in external jugular blood was lower than in rats with their nerves intact, but this difference might possibly be due to the altered pattern of blood-pressure changes during haemorrhage rather than the destruction of nervous connexions between baroreceptors and the pituitary.

The second increase in antidiuretic hormone concentration in blood usually took place when the blood pressure had been less than 50 mm. Hg for about 15 min. Ferritin and adenosine triphosphate liberate antidiuretic hormone from the neurohypophysis (Baez, Mazur, and Shorr, 1952; Dexter, Stoner, and Green, 1954), and it is tempting to suggest that increased secretion of antidiuretic hormone follows the release of these substances from anoxic liver and muscle.

Friedan and Kellar (1954) have found that to lower the blood pressure of unanaesthetized dogs to 70 mm. Hg it is necessary to withdraw more blood from normal animals than from dogs with experimental diabetes insipidus. In rats anaesthetized with pentobarbitone the withdrawal of blood usually produced a sudden fall in blood pressure to below 70 mm. Hg in both normal and neurohypophysectomized animals. However, it was noted that in rats with intact neurohypophyses the blood pressure, after its collapse, remained at about 35 mm. Hg, whereas in the neurohypophysectomized animals it fell to below 20 mm. Hg. This observation is similar to that of Friedan and Kellar (1954), and, together with our other findings, supports their suggestion that the neurohypophysial pressor principle serves a physiological function in maintaining blood pressure during haemorrhage.

The absence of detectable antidiuretic activity in external jugular blood in rats anaesthetized with ethanol or chloralose (before haemorrhage) agrees with observations of Ames and van Dyke (1952) and Dicker (1953), and with expectation based on the continuance of water diuresis during anaesthesia with these drugs. However, the description of ethanol anaesthesia as a state of "functional neurohypophysectomy" (Dicker, 1954) does not apply as far as the stimulation of the posterior pituitary by haemorrhage is concerned. In contrast to ethanol,

anaesthesia with chloralose did suppress the liberation of antidiuretic hormone during haemorrhage; how this was achieved is not clear at present.

The highest antidiuretic activities found in 1 ml. of blood were equivalent to 2–3% of the vasopressin content of the neurohypophysis. However, the neurohypophyses of rats bled under ether, ethanol, and urethane anaesthesia did not have lower hormone contents than those of the control anaesthetized rats; and in rats anaesthetized with pentobarbitone the glands had significantly greater oxytocic and vasopressor activities after haemorrhage. Since haemorrhage under these conditions undoubtedly leads to the liberation of antidiuretic hormones into the blood, this finding can only be explained by increased repletion of the hormones stored in the gland. It may be necessary therefore to distinguish between *tropic* stimuli which affect the posterior lobe so that the hormones are transferred in increased amounts from the gland into the blood, and *trophic* stimuli which increase the amounts of biologically active material in the gland. Ames and van Dyke (1950) found increased antidiuretic activity in the posterior pituitaries of rats which had been deprived of fluid for 3 days, but in most investigations the hormone content of the neurohypophysis has been found to decrease under conditions or after treatments which would increase liberation of hormones from the gland. However, close examination of some of these results leads to the conclusion that trophic stimulation of the neurohypophysial-hypothalamic system also occurred. The normal rate of antidiuretic hormone secretion has been estimated in dogs to be of the order of 1–5 mU./hr. (Shannon, 1942) and 0.3–1.2 mU./hr. in rats (Dicker, 1954); assuming that the vasopressin content of the posterior lobe does not vary greatly from day to day, this rate must be approximately equal to that at which the hormone store is replenished. In Hild and Zetler's (1953) experiments, after restoration of access to water to dogs which had been thirsted for 14 days, the vasopressor potency of neurohypophyses increased at a rate of 2.8 units/day, i.e. the rate of repletion was about 20–100 times greater than normal. More striking perhaps are the results of Dexter *et al.* (1954), who showed that, following depletion by ATP injection, the antidiuretic activity of rat posterior pituitaries was restored by over 200 mU. (one third of the content of a normal gland) in 50 min., i.e. 330–1,300 times faster than the normal rate. Similarly, Rothballer (1953) found that painful stimuli in rats caused almost complete depletion of neurosecretory substance in the neurohypophysis within a few minutes; after 2 hr. the normal content of neurosecretory material was restored. Since the activity in the hypothalamus is small compared with that in the pituitary, repletion at such rates cannot be explained solely on the basis of increased rate of flow of the axoplasm current (Scharrer and Scharrer, 1954). Such stimuli must also increase the rate at which active material is formed in hypothalamic nuclei. The amounts of active substances in the neurohypophysis are determined by the rate of repletion as well as by the rate of hormone release, and the turnover of hormones by the gland may be independent of the level at which they are stored. Changes in the hormone content of the gland are therefore not reliable as an index of secretion.

The ratio of pressor to oxytocic activity in the neurohypophysis was not affected by haemorrhage, but it was altered by some anaesthetics. In round figures the relative proportion of pressor to oxytocic activity (expressed in terms of the appropriate international units) changed from 1 : 1 in unanaesthetized rats to 2 : 1 in rats anaesthetized with pentobarbitone, and to 1 : 2 in rats anaesthetized with urethane. The latter result is interesting, since Macaulay (1950) and van Dyke, Adamson, and Engel (1955) have stated that no instance of a vasopressin-oxytocin ratio of less than unity can be discovered.

Summary

1. In rats anaesthetized with ether, pentobarbitone, or urethane, the antidiuretic activity in external jugular blood before haemorrhage was 0.1–0.25 mU. vasopressin/ml.; it rose to 7–25 mU./ml. after the withdrawal of 2.5 ml. blood/100 g. body weight over a period of 20 min.

2. In rats anaesthetized with ethanol, the initial antidiuretic activity in external jugular blood was less than 0.1 mU./ml.; it rose to 5 mU./ml. or more, after withdrawal of 2.5 ml. blood/100 g. body weight.

3. In rats anaesthetized with chloralose the antidiuretic activity in external jugular blood was too low for detection both before and after haemorrhage, except in one animal where 1.6 mU./ml. was found after withdrawal of 2.5 ml./100 g. body weight.

4. Section of the vagi or denervation of the carotid sinuses did not prevent the increase in antidiuretic activity in the blood during haemorrhage.

5. No antidiuretic activity was found in the blood of neurohypophysectomized rats even after withdrawal of 3 ml. blood/100 g. body weight.

6. The results suggest that during haemorrhage the stimulation of the neurohypophysis to release antidiuretic hormone in increased amounts coincided with a big fall in blood pressure. After the blood pressure had been in the region of 35 mm. Hg for 20 min., a second increase in the antidiuretic activity in blood was observed.

7. Haemorrhage did not change the amounts of oxytocic or vasopressor activities in the neurohypophysis of rats anaesthetized with ether, ethanol, urethane, or chloralose. In rats under pentobarbitone anaesthesia the vasopressor and oxytocic activities in the neurohypophysis were greater in those which had been bled.

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ANTIDIURETIC ACTIVITY OF POSTERIOR PITUITARY PREPARATIONS

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Biological assays depend on the quantitative comparison between the effects of unknown amounts of a biologically active principle and those of a standard preparation. One requirement for a valid assay is that the unknown principle should be qualitatively identical with the standard active principle (Wood, 1946; Finney, 1952). In assays of neurohypophysial activities, commercial preparations from beef pituitaries are usually employed as standards. Such preparations are assayed by the manufacturers and their potencies are expressed in terms of the activity of the international standard pituitary (posterior lobe) powder. In some assays oxytocic activity is used (e.g., in the assay of Pitocin, a preparation of the differentiated oxytocic principle, and of Pituitrin, the undifferentiated extract with both ocytocic and pressor effects), whereas, in other assays, pressor activity is used (e.g., in the assay of Pitressin, a preparation of the differentiated vasopressor and antidiuretic principle).

In a study of the effect of haemorrhage on the active contents of the rat neurohypophysis, extracts of rat posterior pituitaries were assayed for oxytocic, vasopressor, and antidiuretic activity. Since none of the commercial preparations are assayed by antidiuretic activity, and since the extracts to be tested had both vasopressor and oxytocic activities, it was decided, as a precaution, to perform two antidiuretic assays on each extract, one using pitressin as the standard and one with pituitrin. The results of the experiments are described, and the discrepancies observed are discussed, in the present paper.

METHODS

White albino rats of the Wistar strain (180–220 g.) were used in all experiments. Antidiuretic potency was assayed by intravenous injection into unanaesthetized water-loaded rats following the technique of Ginsburg and Heller (1953) as modified by Ginsburg and Brown (1956). Pressor activity was assayed on the blood pressure of rats anaesthetized with urethane and treated with dibenamine as described by Dekanski (1952). Holton's (1948) method was used for the assay of oxytocic activity on the isolated rat uterus.

Rat neurohypophysial extracts were prepared by heating the homogenized glands in a boiling water-bath in 0.025% (w/v) acetic acid for 5 min. Extracts were prepared from the international standard pituitary (posterior lobe) powder following the instructions in the British Pharmacopœia (1953).

Messrs. Parke Davis & Co.'s Pitressin, Pituitrin and Pitocin were also used.

RESULTS

Fig. 1 shows the antidiuretic potencies of rat neurohypophysial extracts compared with their pressor potencies. When pitressin was used as the standard in the antidiuretic assay the antidiuretic potencies were, on average, about $1\frac{1}{2}$ times greater than the pressor potencies, and there was a good statistical correlation between the pressor and antidiuretic activities as they varied from extract to extract ($r = +0.83$; $P < 0.01$). When pituitrin was used as the standard some of the results were in good agreement with those of antidiuretic assays using pitressin as standard, whereas others were 4–6 times greater; the antidiuretic activities of the extracts did not appear to be correlated with the pressor potencies ($r = +0.50$; $P > 0.1$).

The antidiuretic effects of pitressin and pituitrin were then compared directly on the same rats. In ten rats out of sixteen, the difference between the antidiuretic activities of pitressin and pituitrin was within the large error of the method of assay (approx. $\pm 40\%$; Ginsburg and Heller, 1953). In the remaining six rats the antidiuretic potency of pituitrin varied from one half to less than one tenth of the potency of pitressin. Fig. 2 shows an experiment in a rat in which pituitrin had only one ninth of the antidiuretic activity of pitressin. This difference cannot be attributed to the oxytocin content of pituitrin as suggested by Fraser (1937, 1942) and Selye (1949), since the addition of pitocin to pitressin did not affect the antidiuretic response.

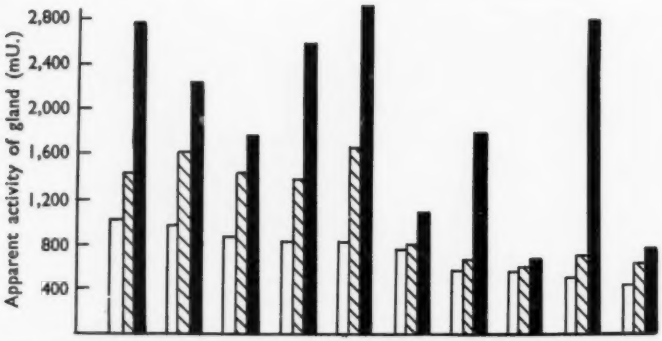

FIG. 1.—Estimated antidiuretic and pressor potencies of rat neurohypophyses. Unshaded columns, pressor potency–pitressin standard. Hatched columns, antidiuretic potency–pitressin standard. Solid columns, antidiuretic potency–pituitrin standard.

In further experiments, pitressin and pituitrin were compared with international standard pituitary (posterior lobe) powder (Fig. 3). The oxytocic potency found for pituitrin agreed well with the manufacturers' estimate, and its vasopressor activity (per unit oxytocin potency) was equal to that of the international standard. However, the antidiuretic activity of pituitrin (compared with the international standard) did not correspond with its oxytocic and vasopressor potencies. In two of the three rats used for these antidiuretic assays pituitrin had less than one third of the activity of the international standard; in the other rat the difference between the antidiuretic potency of pituitrin and the international standard could be within the error of the antidiuretic assay.

Pitressin had the pressor activity claimed by the manufacturers, and, although its antidiuretic potency was less than that of the international standard, the differences were within the error of the antidiuretic assay. These assays were performed on the same rats as had been used for comparing the antidiuretic potency of the international standard and pituitrin. In the two rats in which the antidiuretic effect of pituitrin did not correspond with its vasopressor activity, the antidiuretic potency of pitressin was not less than was expected from its vasopressor activity.

It is well known that corticotrophin preparations are frequently contaminated with posterior pituitary hormones. If corticotrophin were to interfere with the antidiuretic action but not the vasopressor action of posterior pituitary hormone, it is possible that corticotrophin contamination of pituitrin could account for the relatively small antidiuretic effect of pituitrin in some rats. A preparation containing 12.6 units corticotrophin/mg. supplied by Organon Ltd. was tested for antidiuretic and pressor potency. The rats used in the antidiuretic assay had been previously shown to be approximately three times more sensitive to the international standard than to pituitrin. There was good agreement between the results of the pressor and antidiuretic assays, there being 9.6 mU. of vasopressin activity and 8.9 mU. antidiuretic activity in each mg.

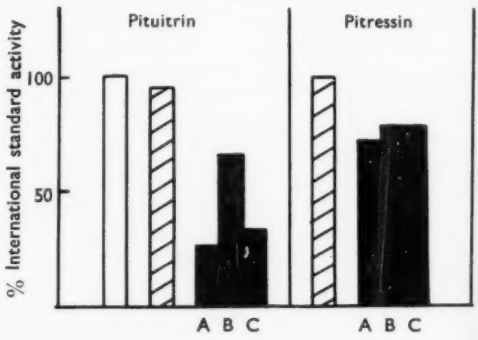

FIG. 3.—Oxytocic, pressor and antidiuretic activities of pituitrin and pitressin expressed as % of the activity of international standard extract. In the antidiuretic tests the same rats, A, B, and C, were used in experiments with pituitrin and pitressin. Unshaded columns, oxytocic activity. Hatched columns, pressor activity. Solid columns, antidiuretic activity.

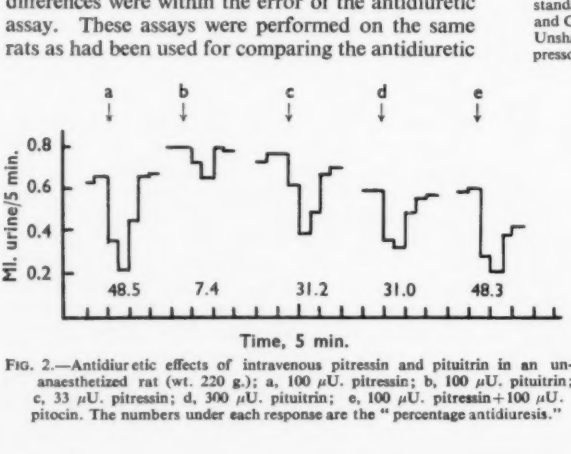

FIG. 2.—Antidiuretic effects of intravenous pitressin and pituitrin in an unanaesthetized rat (wt. 220 g.); a, 100 μU. pitressin; b, 100 μU. pituitrin; c, 33 μU. pitressin; d, 300 μU. pituitrin; e, 100 μU. pitressin+100 μU. pitocin. The numbers under each response are the "percentage antidiuresis."

(international standard was used as standard in these experiments).

Discussion

The problem which prompted this small investigation was to explain why antidiuretic assays of rat neurohypophysial extracts gave higher values when the standard was pituitrin than when pitressin was the standard. The explanation found from the present experiments is that pituitrin seems to have less antidiuretic activity relative to its oxytocic and vasopressor potencies than does either international standard pituitary (posterior lobe) extract or pitressin, a preparation of the differentiated vasopressor principle. This difference between antidiuretic action of pituitrin on one hand, and pitressin and international standard on the other, seems to depend on the rat used for the antidiuretic assay. In about 60% of the rats, the difference was not greater than could be accounted for by the error of the assay ($\pm 40\%$), but in others the difference was sometimes as much as (or more than) tenfold.

These results suggest that there is some factor in pituitrin which can antagonize the antidiuretic effect, and to which rats have varying sensitivities. Oxytocin preparations antagonize antidiuretic effects of vasopressin preparations in some amphibia (Barker-Jorgenson, 1950; Ewer, 1951) and it has been suggested that this may also occur in mammals (Fraser, 1937 and 1942; Selye, 1949). However, in the present experiments, addition of pitocin to pitressin did not affect the antidiuretic response, and international standard (which contains the oxytocic principle) had greater antidiuretic effects than pituitrin. The other possible contaminant excluded by these experiments is adrenocorticotrophic hormone, and it therefore seems unlikely that the corticotrophin releasing activity which contaminates commercial pituitary preparations (Guillemin and Hearn, 1955) can be involved.

Heller and Stephenson (1950) found that in rats pituitrin increased urinary excretion of Na and Cl to a greater extent than could be explained by regarding it as a simple mixture of pitocin and pitressin. Increased excretion of electrolytes could interfere with antidiuretic action, and this effect of pituitrin might account for its relative lack of antidiuretic effect in some rats.

In the present experiments the only commercial neurohypophysial preparations used were pitressin and pituitrin and only one method of testing for antidiuretic activity was employed. It is possible that the discrepancies appear only under these conditions. Nevertheless the implications of these experiments carry the warning that, before a commercial preparation, which may have been assayed by the manufacturer for oxytocic and vasopressor potencies, is used as the standard in antidiuretic assays, it is necessary to ensure that its antidiuretic potency relative to vasopressor potency is the same as in the international standard.

To insist on complete qualitative identity between active principles in standard and unknown in biological assays must be regarded as a counsel of perfection which is often unattainable. In view of the different molecular structures of beef and pig vasopressin (Popenoe, Lawler, and du Vigneaud, 1952) it is unlikely that the active neurohypophysial principles extracted from the pituitaries, other tissues or body fluids in other species are qualitatively identical with beef vasopressin, which is the active principle in international standard and in the commercial preparations. Although there is no reasonable alternative to the use of these beef pituitary extracts as standards it should not be forgotten that, strictly speaking, many assays may be invalid.

Summary

1. Extracts of rat neurohypophysis had greater antidiuretic potencies when pituitrin was the standard in antidiuretic assays than when pitressin was the standard.

2. In 40% of rats used for antidiuretic assays the activity of pituitrin was less than half that of pitressin or international standard.

3. Estimates of the pressor and oxytocic potencies of pituitrin in comparison with international standard and pitressin were in good agreement with those of the manufacturers.

4. The lower antidiuretic potency of pituitrin cannot be attributed to its oxytocin content or to corticotrophin or to corticotrophin-releasing activity contaminating pituitrin.

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THE SPERM-SHEDDING RESPONSE OF MALE TOADS AND TREEFROGS AFTER THE INJECTION OF TWO TYPES OF GONADOTROPHIN

BY

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Injection of gonadotrophin into the male British toad (*Bufo bufo*) is followed by the shedding of sperm, the response being of the normal quantal type (Frazer and Wohlzogen, 1951). The dose-response curve of the toad has now been investigated more fully with chorionic gonadotrophin, which is of the luteinizing type. At the same time the response to pregnant mare's serum gonadotrophin (of the follicle-stimulating type) was also studied to see if the two curves differed greatly in either ED50 or slope. The response of the treefrog (*Hyla arborea*) was also investigated, since it seemed possible that this smaller species might respond to smaller doses, and so be more suitable for the estimation of relatively small amounts of gonadotrophin.

The effect of chorionic gonadotrophin on the toad was also examined to see whether it altered at different times of the year under relatively standard laboratory conditions. The effect of alteration in environmental temperature was also studied. With this knowledge, the gonadotrophin excretion during pregnancy was followed in 5 cases, using a technique already developed (Frazer and Wohlzogen, 1951).

METHODS

Experimental Animals.—Toads and treefrogs were kept in an animal house at approximately 21.5° C. The experiments were normally conducted in a laboratory kept at about 20° C. The conditions during the temperature experiments are described later. In the seasonal work, the batches of toads were taken as far as possible through the summer at 6-week intervals, being used exactly one week after they had been obtained from the dealer. The interval between receipt and use was left to avoid using toads which might already have been shedding sperms. The isolation of the males from females for a week is normally sufficient to achieve this object, even when, on receipt from the breeding ponds, the cloacal urine of the males is swarming with spermatozoa. They were kept during this week in large cages containing moss and water, and fed on maggots and flies.

The toads were from the south of England, and the treefrogs were of sp. *typica* from N. Italy.

Gonadotrophins.—International Standard gonadotrophins were used for the dose-response curves, the luteinizing type from human pregnancy urine (UP) and the follicle-stimulating type from pregnant mares' serum (PMS); for the experiments on temperature, a crystalline preparation was made from pregnancy urine by the method of Claesson, Hogberg, Rosenberg, and Westman (1948). The gonadotrophin was always dissolved in distilled water; the injection volume was 1 ml.

When gonadotrophins were being estimated in pregnancy urine, the urine was filtered and injected directly. Dilutions of urine were also used, and gonadotrophin concentrates were made from samples when necessary, either because of the toxicity of the urine or because there was only a low level of gonadotrophin in it. For this purpose, the method of Scott (1940) was used, modified by the use of Universal Indicator (B.D.H.). The technique of Frazer and Wohlzogen (1951) was used for gonadotrophin estimation.

Experimental Techniques.—All injections were made into the dorsal lymph sac of toads or treefrogs whose urine was free of spermatozoa. The method has already been described, as well as the technique of sampling toad's urine (Frazer, 1950). Cloacal urine was taken 3 hr. after injection, and the presence or absence of spermatozoa noted.

At all times the amphibians were handled gently, so as to minimize struggling. If, in spite of these precautions, there was some reflux of injected fluid, the animal was rejected and another one taken. This was necessary, since the loss of one drop (0.05 ml.) represented a loss of 5% of the total given.

RESULTS

Dose-response Curves.—Tables I and II give the results with International Standard gonadotrophins, the corresponding dose-response curves being plotted in Fig. 1. Table II shows no appreciable

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TABLE I

SPERM-SHEDDING RESPONSE OF TOADS AND TREEFROGS AFTER INTERNATIONAL STANDARD GONADOTROPHIN (UP AND PMS)

Species	Gonadotrophin	Dose (i.u.)	No. Positive	No. in Group
B. bufo ..	UP	4	12	155
		6	44	130
		7·5	61	130
		9·375	67	110
	PMS	20	2	30
		22	9	30
		24	22	30
H. arborea	UP	1	20	50
		1·5	36	50
		2·5	46	50
	PMS	2·5	8	36
		3·5	12	25
		5	30	36

TABLE II

SPERM-SHEDDING RESPONSES OF MALE TOADS AT DIFFERENT SEASONS

The smallness of the groups in October and January was due to difficulty in supply. For the same reason, the January results were obtained with the survivors of the October experiments. Otherwise, fresh batches of toads were used for each experiment

Season	Number of Toads at Each Dose	Number of Toads Responding		
		6 i.u.	7·5 i.u.	9·375 i.u.
April ..	30	9	10	17
May–June	30	11	15	18
July ..	30	11	16	21
October ..	20	5	10	11
January ..	20	8	10	—

seasonal variation, so results obtained with 4 i.u. UP on toads during March and April have been included in Table I. It has thus been possible to find a characteristic curve for use with pregnancy urine throughout the year.

Gonadotrophin Content of Pregnancy Urine.—This was calculated at intervals during the pregnancy of 5 subjects (Frazer, 1954a), the equation of Fig. 1 being used to measure the 24-hour output. The method was devised to give 95% confidence limits, and these are given for a typical case in Fig. 2, where the means have been joined up to form a curve.

Effect of Temperature.—Although the findings under standard laboratory conditions had shown no significant seasonal variation in response, experiments with the crystalline preparation at varying laboratory temperatures gave:

0/12 positive at 5° C.; 28/39 positive at 16–17° C.; 39/40 positive at 22–24° C.

Further, 14 toads treated at 22° C. all showed only moderate numbers of sperms, whereas all save one

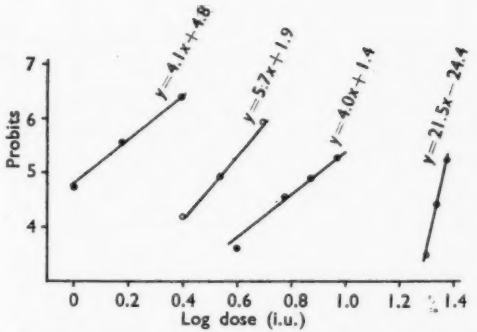

FIG. 1.—Dose-response curves for *B. bufo* (on right) and *H. arborea* (on left) when injected with gonadotrophins. The solid circles give responses to UP and the hollow circles to PMS. The formulae from which the lines were drawn are given.

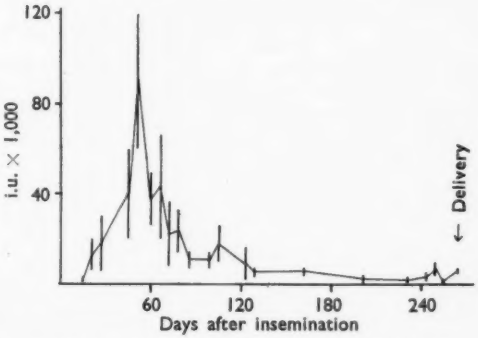

FIG. 2.—Day to day 24-hr. gonadotrophin outputs from a typical human pregnancy. The vertical lines indicate the 95% confidence limits.

of a similar group at 26° C. had numerous sperms in their urine.

Apart from the absence of sperm shedding at 5° C., keeping these toads for a further three hours at 20° C. only resulted in five positives, against a control group at 20° C. throughout, which showed 11 positives out of 12 in three hours. This slowness of response, even after the low temperature had been succeeded by the normal one, led to two further batches of 12 toads being placed at 5° C. overnight, followed by 20° C. in the morning. After an interval of 1½ hours, to allow their body temperature to return to the normal in that environment, each group was injected with the same mixture. One group was then kept at 5° C. and one at 20° C. In the next three hours, no positive response occurred in either group, but after 24 hours 5 in each group showed spermuria.

Discussion

The results given in Fig. 1 show that it is possible to obtain regular curves relating the dose of gonadotrophin to the sperm-shedding response of the toad and treefrog. They were obtained under relatively stable experimental conditions. It is noticeable that the slopes of the two curves for the response of *B. bufo* differ from one another. Yet Wohlzogen (1954), using this species, found the same slope for both types of gonadotrophin. A possible explanation may be that the two lots of toads receiving PMS were treated at different times of year; but there is at present no evidence on seasonal variation in the response to this type of gonadotrophin. Alternatively, some slight difference in the conditions of housing or of the experiments might have been responsible. Apart from this one curve, the slopes of the other three are remarkably alike, and are comparable also with that originally obtained by Winston (1952, personal communication) in *B. regularis*, where he found that the equation $y=4.04x-0.76$ applied to the first 60 toads he used. It is possible that the greater variation in his later findings may have been due to the much greater variation in the size of *B. regularis* males, which may be associated with greater variability in response: in *B. bufo*, it has been shown by Wohlzogen (1954) that light toads are more sensitive than heavy ones. This latter fact may also account for the present slope of 4.0 found for the response of the latter species to UP, which differs markedly from that originally found (Frazer and Wohlzogen, 1951). The ED50 has remained unaltered since the earlier work.

Both the present species show variation in the ED50 for their response to UP and PMS, which can be accounted for by the difference in the actual amount of each gonadotrophin defined as equivalent to an International Unit. Wohlzogen (1954) found corresponding responses in toads when 3.5 times as many International Units of PMS as of UP were used.

Comparison of the results shows that the treefrog was 7–8 times as sensitive to UP as the toad. Since the former can only be readily obtained in adequate numbers during the early summer months, this increased sensitivity is not enough to warrant its use throughout the year in place of the toad, though it may occasionally be a useful replacement.

While the work on treefrogs was taking place, Wohlzogen and Halama (1951) were performing a similar investigation with UP in Vienna, the only difference being that their treefrogs came from Eastern Austria. Comparison between the two sets of work (Frazer and Wohlzogen, 1953) showed that the slope of the regression line could be taken as common to both investigations with a value of 3.88, although there was a distinct variation between them as far as the ED50 was concerned: the Austrian treefrogs were the more sensitive. Using this value for the slope (to the nearest decimal place), together with an ED50 of 1.13 i.u., the equation for the response of treefrogs in England becomes

$$y=3.9x+4.8$$

This line is almost identical with that shown in Fig. 1.

Under the standard conditions, no seasonal variation in response was seen, but it was later shown that there was a change in threshold so slight as not to be normally perceptible. During 1952 the response to UP given three hours after a subthreshold dose was greater than if both doses were given simultaneously (Frazer, 1954b). But about the beginning of May there was a marked drop in response: during 1953 a similar falling-off was noticed (see Table III), so that it is now apparent that at the end of the toad's normal breeding season the response is slightly reduced, although this lowering of sensitivity is not detectable with the ordinary technique.

Table III

SEASONAL VARIATION IN THE RESPONSE OF *B. BUFO* TO INTERNATIONAL STANDARD CHORIONIC GONADOTROPHIN (UP), WHEN GIVEN IN TWO DOSES AT 3-HR. INTERVALS

Dose (i.u.)	Numbers Positive Against Numbers Injected	
	1 Hr. After 2nd Dose	3 Hr. After 2nd
A. Before mid-April		
4+1	0/30	5/30
4+2	8/30	24/30
4+2·5	6/11	8/11
B. After mid-April		
4+1·5	0/20	0/20
4+2	0/30	1/30
4+2·5	1/37	4/37

With the usual standard conditions, the results obtained on samples of pregnancy urine proved perfectly satisfactory and were in agreement with the findings of previous workers (Evans, Kohls, and Wonder, 1937; Loraine, 1950). The method would also be applicable to estimations of the level of serum gonadotrophin in pregnancy, although care must be taken not to use sera toxic to the toads. Allison (1954) has overcome this by the modification of injecting a smaller amount of serum mixed with hyaluronidase, and has obtained satisfactory responses. Gastineau, Albert, and Randall (1948, 1949) have shown that the renal clearance of

gonadotrophin is relatively constant, so that the serum levels will be reflected in the urinary ones.

Table II shows no variation in the response of *B. bufo* to UP during one year's work. Similar experiments were being performed on the same days by Wohlzogen, but using *Bufo viridis*. He found (1953, private communication) that the slope remained unaltered in this species, but that there was variation in ED50. His toads had been caught a week before use, but had been stored meanwhile in a cool cellar. Thus, in neither species was there variation in the slope of the line with season: yet *B. bufo* kept at 21.5° C. showed no change in ED50 whereas *B. viridis* kept cool before use showed such a change. It was possible that either the species difference or the variation in local conditions was affecting the response.

Although the response of *B. bufo* to UP has been shown not to alter with season under standard conditions, a very great difference occurred between the response at 16–18° C. and that at markedly higher or lower temperatures; and temperatures as low as 5° C., before or during the experiment, would inhibit the response normally expected at 22° C. Houssay (1947) in *Bufo arenarum* obtained a smaller response at 3° C. than at 26° C. Jeffree (1953) has reported that *B. bufo* kept in an unwarmed room showed greater sensitivity between March and August, which is again suggestive of the temperature effect reported here. These findings make it necessary to stress that any work where constancy of response is required should be carried out under standard conditions of temperature, both during and before the experiment.

Summary

1. The sperm-shedding responses of the male toad (*Bufo bufo*) and treefrog (*Hyla arborea*) to International Standard gonadotrophins in water have been studied. The dose-response curves of both species with gonadotrophins of both follicle-stimulating and luteinizing types have been plotted, and the formulae of the regression lines which fit these points worked out.

2. Under standard laboratory conditions, the curve when toads were given gonadotrophins from pregnancy urine was fitted at all seasons by the regression line of formula $y=4.0x+1.3$.

3. The response of toads to chorionic gonadotrophin varied with the temperature at which the experiment was performed.

4. It is stressed that regular responses can only be obtained under standard conditions.

I am indebted to the Department of Biological Standards of the Medical Research Council for kindly making available the supply of International Standard gonadotrophins.

The work in this paper formed part of a thesis accepted by the University of Oxford for the degree of Doctor of Medicine.

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EXPERIMENTAL DESIGN FOR BIOASSAY OF A MATERIAL INDUCING STRONG TACHYPHYLACTIC EFFECT (ANAPHYLATOXIN)

BY

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Anaphylatoxin, prepared by incubation of rat plasma with agar or starch, stimulates guinea-pig ileum by releasing histamine (Rocha e Silva and Aronson, 1952; Rocha e Silva, 1952a; Rothschild and Rocha e Silva, 1954). The effect is therefore indirect, and repetition of the same dose of activated plasma produces less and less effect until the preparation is desensitized to it. This tachyphylactic effect, though strong, is progressive and bound to follow certain rules, if the assay is done in an accurate way. However, the possibility of comparing two different preparations of anaphylatoxin by applying them alternately to the same piece of intestine would give very inaccurate results on account of the enormous bias introduced by the tachyphylactic effect. Even with randomized blocks as utilized for histamine (Schild, 1942), oxytocin (Holton, 1948) or bradykinin (Rocha e Silva, 1952b), the bias introduced by the tachyphylactic effect of anaphylatoxin is so strong that no accurate potency ratio could be derived. The responses depend greatly upon the position of the dose in the sequence of treatments. For instance, the response to the first dose can be 20 times that of the same dose applied after several treatments. Moreover, one of the prerequisites for a sound bioassay has been supposed to be the independence of all responses on the same animal or preparation. However, the literature shows the possibility of treatment comparisons even where a correlation between responses might be expected (Finney, 1955). Following a suggestion by Finney (1955) we have tried several designs in order to test the possibility of setting up a (2+2) assay for a quantitative estimation of anaphylatoxin. Much to our surprise, a Latin square design in which columns were separate pieces of gut, appeared to correct for the bias introduced by the tachyphylactic effect, allowing a reasonable estimate of the ratio of potency between "standard" and "unknown."

The possibility of segregating a sum of squares corresponding to the tachyphylactic effect affords a useful means of studying phenomena such as those occurring in anaphylaxis, or as a consequence of the action of animal venoms or histamine releasers, in which the pharmacological effects depend upon the liberation of active substances from an exhaustible stock bound to the tissues of the organs involved. Other analogous phenomena, such as the hypertensive effect produced by the release of hypertensin following the injection of renin, could possibly be studied more accurately by employing the experimental design described in this paper.

METHODS AND DESIGN

Biological Preparation.—Pieces of intestine from a bled guinea-pig were washed with Tyrode solution and distributed in separate beakers containing Tyrode solution. After storage in the ice-box for varying times, the pieces were suspended in a 10 ml. chamber and the experiments started after 10 min. To test the sensitivity of the gut, a standard dose of histamine was added a few times, and then the four doses of anaphylatoxin were added at intervals of 3 min. Four different doses of anaphylatoxin were assayed upon the same fragment, forming one "Group." The sequence of additions was that indicated in the next paragraph. The anaphylatoxin solutions were prepared as described previously (Rothschild and Rocha e Silva, 1954) by adding an optimum amount of agar to a heparinized sample of rat plasma. After incubation for 30 min., the material was centrifuged and kept frozen until used. In the following experiments "standard" and "unknown" are dilutions of the same plasma. All other conditions of the assay are the same as those described in previous publications.

Experimental Design.—Any attempt to devise a (2+2) assay for potency estimations, using a material that stimulates guinea-pig ileum, should aim at obtaining the responses to the four doses of standard and unknown (S_1, S_2, T_1, and T_2) in a single piece of ileum. Fortunately, desensitization (tachyphylaxis) to anaphylatoxin

is sufficiently slow to allow a complete sequence of four treatments to be assayed upon the same piece of ileum. It became, therefore, possible to confound "inter-intestines" variation with group variation, so that the potency estimation could be derived from "intra-intestines" comparisons. Similarly, by employing the Latin square design, the tachyphylactic effect was confounded with the "order of additions" (rows). By so doing the random error became reasonably low when compared with many biological assays—such as those for vitamins or insulin.

Although several Latin squares have been used most of the experiments were done with the following standard square:

Groups: I	II	III	IV
A	B	C	D
B	A	D	C
C	D	A	B
D	C	B	A

Treatments were allocated to the letters thus: $A=S_1$, $B=S_2$, $C=T_1$, $D=T_2$, where S_1 and S_2 indicate the lower and higher doses of the "standard," and T_1 and T_2 the corresponding doses of the "unknown." Although in the present experiments treatments have not been randomly allotted to the letters for each assay, such a procedure would be advisable for routine assays. The ratio $S_2/S_1=T_2/T_1$ was 2.00, unless otherwise indicated, and the ratio $T_1/S_1=T_2/S_2$ was arbitrarily made 1.5 or 1.00, as indicated.

A piece of ileum taken randomly from one guinea-pig was allotted to each group of a square; each square was thus performed with four pieces of gut from a single guinea-pig.

If we represent the totals for each dose by the corresponding letters S_1, S_2, T_1, and T_2, the three important contrasts:

$$\theta=-S_1-S_2+T_1+T_2$$
$$\varphi=-S_1+S_2-T_1+T_2$$
$$\psi=+S_1-S_2-T_1+T_2$$

will permit the calculation of the three parts into which the sum of squares corresponding to the variation due to "Doses" can be split: "Between samples" $=\theta^2/16$, "Slope" $=\varphi^2/16$ and "Deviation from parallelism" $=\psi^2/16$, each with one degree of freedom. The mean squares "Between samples" and "Slope" are also indicated by the two squares D^2 and B^2, respectively. The validity of the assay corresponds to an expectation of $\psi=0$, or to a variance ratio $\psi^2/16s^2$ smaller than 5.99 (F) at a probability level of 5%, and 6 degrees of freedom. Contrariwise, the variance $\varphi^2/16=B^2$ must be as large as possible when compared with the Error variance (s^2) of the assay. The value of $\theta^2/16=D^2$ will depend upon the artificial conditions of the dummy assay, in which the ratio "Unknown"/"Standard" is arbitrarily chosen. This ratio was taken as 1.50 in the present experiments except in one (Expt. 3, Table III) where it was 1.00.

The first two contrasts presented above permit the calculation of the logarithmic ratio of potency (M):

$$M=\frac{I\theta}{\varphi}=\frac{ID}{B}$$

where $I=\log(S_2/S_1)=0.301$. In Expt. 2 of Table III, $I=0.352$ because the ratio between the larger and the smaller doses of the standard, and those of the unknown, was 2.25. Likewise, the slope of the mean regression line will be given by the formula: $b=2\varphi/16\,I$ and the variance of the slope $s_b^2=2s^2/16\,I^2$. The standard deviation in terms of the log dose, indicated by $\lambda=s/b$, can measure the efficiency of the assay (Gaddum, 1941); alternatively, the reciprocal of its square ($1/\lambda^2$) could be taken as a measure of the "inherent precision" of the assay.

For the calculation of the error of M, the simplified formula

$$s_M=\frac{sI\sqrt{B^2+D^2}}{B^2}$$

was used and the fiducial limits of the ratio of potency calculated in the conventional way, by taking the antilogs ($M\pm s_M t$), where $t=2.45$ for a probability level of 5% and 6 degrees of freedom (Fisher and Yates, 1943). When the ratio b/s_b was lower than 7.8, the exact formula for s_M was employed (Bliss, 1952) and the fiducial limits calculated by the formula:

$$CM\pm ts_M\sqrt{C}$$

where $C=B^2/(B^2-s^2t^2)$.

The more elaborate calculation of S_M and the fiducial limits was applied to Square IV, Expt. 1; Squares II and III, Expt. 2; and Squares I and II, Expt. 3, of Table III.

For the calculation of the weighted mean, $\bar{M}$, and the corresponding $s_{\bar{M}}$, as well as the number of degrees of freedom in $s_{\bar{M}}$ (D.F. in $s_{\bar{M}}$) the schedule indicated by Bliss (1952, p. 579) was applied. The test of homogeneity of variances was Bartlett's test as described by Snedecor (1946). In general, the notation adopted in this paper is that given by Emmens (1948) and Finney (1952).

For the test of linearity, a Latin square design was also adopted, but the ratio of successive doses was 1.50. The splitting of the sum of squares corresponding to "Doses" was performed by utilizing the polynomial coefficients for doses.

In each experiment, to test the linearity, four squares were assayed in a sequence, and the 4×4 pieces of intestine of four different guinea-pigs (one guinea-pig for each square) were randomized as described in another section (Results). Attempts to cut as nearly identical pieces as possible were seldom successful, but it does not appear to be essential for the design of the assay.

Coefficient of Desensitization (Δ).—The simplest assumption about the mechanism of tachyphylaxis is that each dose (A, for instance) will affect the responses to any subsequent dose (B, C, or D) by a certain value, Δa, that will reduce the expected responses β, γ, and δ given by the following doses B, C, and D, respectively. The residual effects introduced by the tachyphylactic

phenomenon in the first column of the Latin square presented above would be indicated by the following expressions:

Group I (α), $(\beta-\Delta\alpha)$, $(\gamma-\Delta\alpha-\Delta\beta)$, and $(\delta-\Delta\alpha-\Delta\beta-\Delta\gamma)$,

and similar expressions would be obtained from the other columns of the square.

Now, if the responses are assembled according to the doses, the totals for each dose will be:

$$T_A = 4\alpha - 2\Delta\beta - 2\Delta\gamma - 2\Delta\delta$$
$$T_B = 4\beta - 2\Delta\alpha - 2\Delta\gamma - 2\Delta\delta$$
$$T_C = 4\gamma - 2\Delta\alpha - 2\Delta\beta - 2\Delta\delta$$
$$T_D = 4\delta - 2\Delta\alpha - 2\Delta\beta - 2\Delta\gamma$$

From the contrasts, (I) $-1\ -1\ +1\ +1$ and (II) $-1\ +1\ -1\ +1$, calculated with these totals, the following expressions are obtained:

$$\text{Contrast (I)}\quad \theta + \frac{\theta\Delta}{2} = \theta\left(1+\frac{\Delta}{2}\right)$$

$$\text{Contrast (II)}\quad \varphi + \frac{\varphi\Delta}{2} = \varphi\left(1+\frac{\Delta}{2}\right)$$

It is therefore obvious that the ratio of the two contrasts I and II, giving the logarithmic ratio of potency (M), is independent of Δ, if the assay is designed as a Latin square. Therefore, M can be calculated directly from the experimental data, by simply applying the formula:

$$M = \frac{I\,\theta}{\varphi} = \frac{I\,[\text{Contrast I}]}{[\text{Contrast II}]}$$

The close agreement between theoretical and experimental M, derived from the assays described below, is a good indication of the reliability of the assumptions involved in the mathematical development described above. The model adopted has been confirmed by the analysis of the experimental results.

Results

Estimates of Potency.—The possibility of deriving a reasonable ratio of potency by applying a (2+2) assay to such apparently variable data as the responses to different doses of anaphylatoxin is a good indication that the tachyphylactic effect is corrected by the experimental design adopted. In the five squares of Expt. 1, Table III, the ratio of potency T/S was made 1.50, by simple dilution of the activated plasma, the undiluted one being taken as " unknown " and the dilute one as " standard."

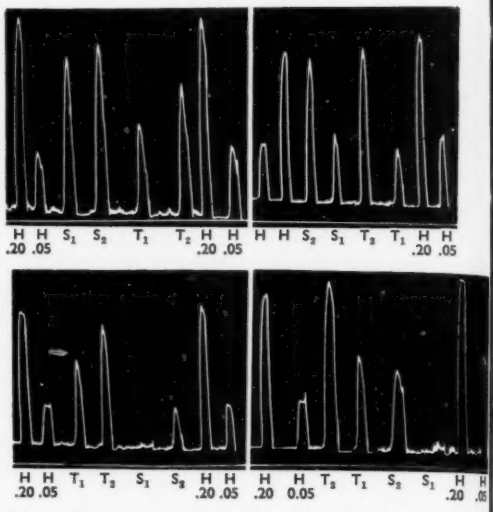

FIG. 1.—Responses of the guinea-pig intestine (four pieces) to four doses of anaphylatoxin. Latin square design for a (2+2) assay. H, histamine solution 1 : 2 millions. S_1, S_2, T_1, and T_2 are the doses of the " standard " and " unknown," respectively.

The data corresponding to square I are presented in detail (Fig. 1 and Table I) and a summary of the five squares is presented in Table III.

Table I shows the responses in millimetres given by the four doses added in the order indicated in (a), where the groups (columns) represent different pieces of ileum and the rows the " order of addition." In (b), the responses have been classified according to " doses."

Simple inspection of Table I shows how strong is the tachyphylactic effect. The smaller dose (S_1) of the standard gives a response of 63 mm. when

TABLE I

RESPONSES (MM.) OF FOUR PIECES OF GUINEA-PIG ILEUM TO DOSES (S_1 AND S_2) OF THE STANDARD AND TO DOSES (T_1 AND T_2) OF THE UNKNOWN

The experimental sequences are given in (a). In (b) the responses are assembled by doses

(a)					(b)				
Groups: I	II	III	IV	Totals T_B	Doses/ Groups	S_1	S_2	T_1	T_2
$S_1=63$ $S_2=69$ $T_1=36$ $T_2=54$	$S_2=58$ $S_1=28$ $T_2=63$ $T_1=22$	$T_1=35$ $T_2=49$ $S_1=2$ $S_2=18$	$T_2=68$ $T_1=39$ $S_2=33$ $S_1=3$	224 185 134 95	I II III IV	63 28 2 3	69 58 16 33	36 22 35 39	54 63 49 68
Totals T_G 222	171	102	143	638	Totals T_D	96	176	132	234

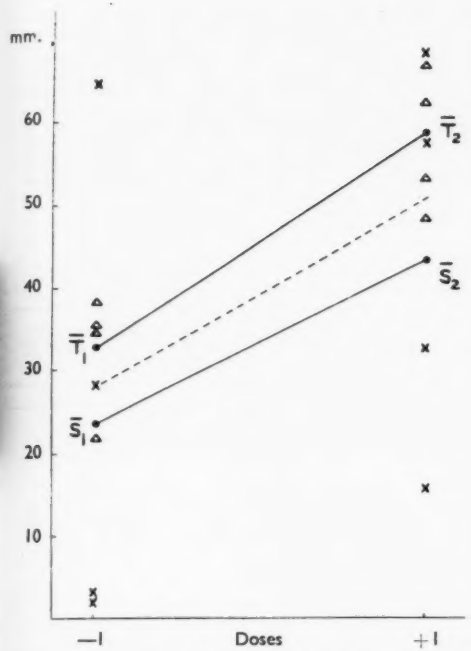

FIG. 2.—Regression lines for unknown (T_1, T_2), standard (S_1, S_2), and mean (dotted line). Individual responses to unknown (△) and standard (x). Abscissae, factorials for doses.

applied first (Group I) and only 3 mm. when applied last (Group IV). The tachyphylactic effect is obviously eliminated from the totals for doses (T_D) from which a reasonable ratio of potency T/S=1.43 could be calculated. Fig. 2 shows the two regression lines for standard and unknown as well as the average regression line, and again it is impressive to note the enormous scatter of the individual data about the means.

By the analysis of variance presented in Table II, the large sums of squares corresponding to variation due to " Groups " and to " Order of additions " (" Rows ") can be isolated and subtracted from the " Total " leaving a residual sum for " Error " that is quite acceptable in this kind of biological assay. The mean square corresponding to " Deviation from parallelism " is of the order of magnitude of the Error variance and therefore non-significant.

Table III presents the most important statistics calculated from the data obtained in the 5 Latin squares of Expt. 1. All but one (Square IV) of the potency ratios fall inside of the fiducial limits, and only once (Square V) was the deviation from parallelism significant at the 5% level.

Expt. 2 of Table III is the same as Expt. 2 of Table VI, in which the four doses were considered as a sequence with an interval of log 1.5=0.176. To derive a ratio of potency, the alternate doses were arbitrarily taken as S_1, S_2, T_1, and T_2, with a ratio T/S=1.5 and $S_2/S_1=T_2/T_1=2.25$.

Expt. 3 of Table III includes four squares in which the ratio T/S=1.00 and the ratio $S_2/S_1=T_2/T_1=2.00$.

Test of Linearity.—The same Latin square design was utilized throughout the following experiments. Two series of 4 squares each were submitted to a test of linearity. The calculation of the theoretical equations was made routinely by using polynomial coefficients (Emmens, 1948).

Randomization of the pieces of guinea-pig ileum was done, as usual, by taking four pieces of intestine from the same guinea-pig and distributing them at random in four beakers containing Tyrode solution and keeping them in the icebox. For the four squares of each experiment, another Latin square design was chosen at random to allocate each fragment to each group of the main design in such a way that no repetition of " age " of the fragment to the same line was allowed.

For the test of linearity, four doses were used: 0.02, 0.03, 0.045, and 0.0675 ml., the constant ratio between the doses being 1.5. This range of doses was well tolerated by the pieces of gut, and good responses were obtained (Table IV).

Table V shows a typical analysis of variance of the data of Table IV, and Fig. 3 shows the theoretical lines (in full) as well as the individual lines corre-

TABLE II

ANALYSIS OF VARIANCE OF THE DATA OF TABLE I

Source of Variation:	Sum of Squares	Degrees of Freedom	Variances	F	P
Total	7,231·75	15			
Between doses: Samples	552·25	1	552·25 (D^2)	12·3	<0·05
Between doses: Regression	2,070·25	1	2,070·25 (B^2)	45·0	<0·01
Between doses: Dev. Parallel	32·50	1	32·50	0·7	
Between groups	1,904·25	3	634·75	14·2	<0·05
Between rows	2,405·25	3	801·75	17·9	<0·01
Error (residual)	267·25	6	44·81		

TABLE III

SUMMARY OF THREE EXPERIMENTS FOR ESTIMATION OF POTENCY BY THE (2+2) ASSAY

Expt.	Square	Ratio (Found)	Slope (b)	Error Variance (s^2)	Stand. Deviation (s)	λ (s/b)	s_M	Fiducial Limits	Variances (Mean Squares)		
									Stand. x Unkn.	Slope	Parallel.
1	I	1·43	74·5	44·80	6·70	0·090	0·050	1·89–1·08	552·25	2,070·25	33·05
	II	1·35	74·2	67·50	8·21	0·111	0·060	1·89–0·96	361·00	1,980·25	56·25
	III	1·34	56·25	18·60	4·32	0·77	0·041	1·70–1·06	203·06	1,139·06	39·06
	IV	1·75	47·70	34·60	5·89	0·123	0·087	3·62–1·18	650·25	900·00	132·25
	V	1·29	63·33	25·70	5·07	0·080	0·043	1·65–1·07	196·00	1,444·00	144·00
	Means:	1·37					0·031	1·59–1·18			
		1·50 (real)									
		$\bar{M}$=0·1365; D.F. in $S_{\bar{M}}$=21.5									
		Homogeneity of variances: χ^2=2·26 0·50<P<0·70									
		Homogeneity of M: χ^2=2·22 0·70<P<0·80									
2	I	1·33	54·1	19·00	4·36	0·079	0·034	1·61–1·10	202·06	1,620 06	10·25
	II	1·51	50·5	74·33	8·62	0·170	0·101	3·83–0·93	324·00	1,260·25	36·00
	III	1·42	61·4	63·17	7·95	0·130	0 072	2·41–0·99	361·00	1,980·50	1·00
	IV	1·39	76·2	29·17	5·40	0·071	0·033	1·67–1·21	663·06	2,943·06	0·06
	Means:	1·37					0·026	1·56–1·21			
		$\bar{M}$=0·1375; D.F. in $s_{\bar{M}}$=14·0									
		Homogeneity of variances: χ^2=2·67 0·30<P<0·50									
		Homogeneity of M: χ^2=1·21 0·70<P<0·80									
3	I	0 91	37 2	102·16	10·10	0·279	0·143	3·14–0·17	18·06	885·06	5·06
	II	1·11	87·0	177·43	13·30	0·153	0·078	2·14–0·62	52·29	2,093·06	52·06
	III	1·19	72·1	17·61	4·20	0·058	0·031	1·42–0·99	105·06	1,743·06	52·56
	IV	0·91	47·1	20·20	4·50	0·095	0·048	1·19–0 69	14·06	798·06	7·53
	Means:	1·09					0·032	1·26–0·92			
		1·00 (real)									
		$\bar{M}$=0·0327; D.F. in $s_{\bar{M}}$=11·6									
		Homogeneity of variances: χ^2=2·286 0·50<P<0·70									
		Homogeneity of M: χ^2=3·01 0·30<P<0·50									

sponding to each group (intestines). It is certainly remarkable that such an enormous variation in the data forming the group sequences corrects itself in the means of the responses to the 16 doses applied in the Latin square design, so as to give a curve that does not deviate significantly from linearity.

In another series of four squares similarly designed, a striking confirmation of the findings in the first experiment was obtained. Fig. 4 presents the four theoretical lines calculated by the least square method and the experimental points corresponding to the means of the responses for each dose. Table VI presents the most relevant data from the 8 squares utilized for the test of linearity, including the respective equations obtained by applying the method of least squares.

Estimate of the Coefficient of Desensitization.— The previous assumption about the mechanism of tachyphylaxis—that each dose will affect the responses to any subsequent dose by a certain residual value ($\Delta\alpha$) that will reduce the expected response to the following dose—would find a reasonable foundation if we assume that each addition will reduce the histamine store of the guinea-pig ileum by a fraction dependent upon the magnitude of the dose previously applied. Since

TABLE IV

EXPERIMENTAL DATA OF THE RESPONSES TO FOUR DOSES OF ANAPHYLATOXIN USING FOUR DIFFERENT PIECES OF ILEUM (GROUPS)

Groups:	I	II	III	IV	Totals T_R	Doses/ Groups	A	B	C	D	Totals T_G
	D 62	A 50	C 62	B 36	210	I	5	17	48	62	132
	C 48	B 46	D 61	A 17	172	II	50	46	52	52	200
	B 17	C 52	A 3	D 39	111	III	3	19	62	61	145
	A 5	D 52	B 19	C 10	86	IV	17	36	10	39	102
Totals: T_G	132	200	145	102	579	Totals: T_D	75	118	172	214	579

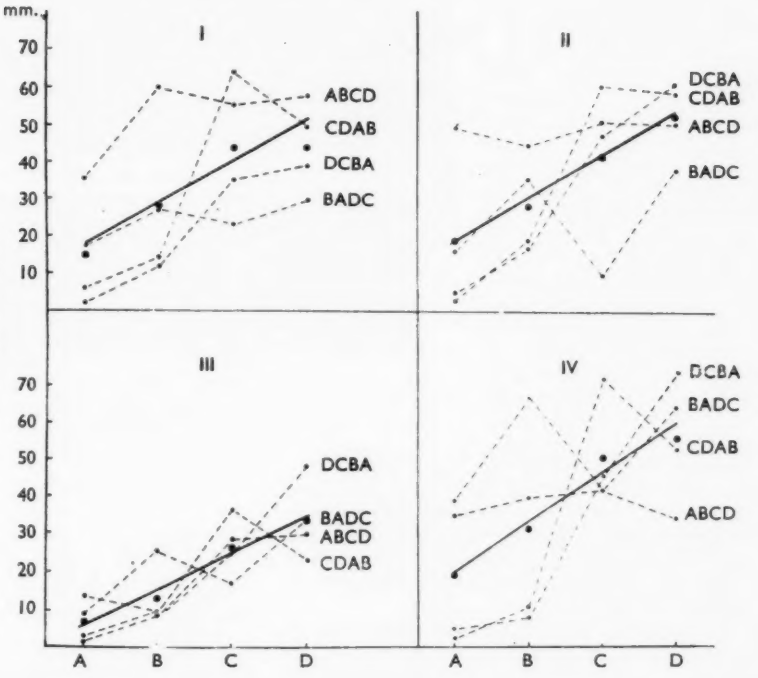

FIG. 3.—Tests of linearity. Dotted lines, responses to treatments upon the same piece of ileum (group). Larger dots: the means to each dose. Solid lines, the theoretical lines calculated by the least squares method.

there is no refilling of the histamine store—at least for the duration of each experiment—we can assume that this residual effect introduced by any previous addition of anaphylatoxin is permanent and cumulative. These assumptions, in good agreement with the experimental results, constitute the basis for the establishment of the model proposed in this paper.

The definition of Δ, the coefficient of desensitization, arises naturally from the model adopted in this paper (Methods and Design). If we take, for instance, the simplest case of repeated additions of the same dose of anaphylatoxin to a single piece of intestine, and if the full effect be represented by Y_0, the first response to that particular dose of anaphylatoxin and the subsequent effects are represented by the responses $Y_1 = Y_0 - \Delta Y_0$, $Y_2 = Y_0 - 2\Delta Y_0$, $Y_3 = Y_0 - 3\Delta Y_0$ and so forth, the series of responses showing a downward trend for each new addition of the same dose of anaphylatoxin. The experiment has shown that this sequence forms a linear regression; for convenience this can be called regression of "responses on the number of previous additions of anaphylatoxin" or of

TABLE V

ANALYSIS OF VARIANCE OF THE DATA OF TABLE IV

Source of Variation:	Sum of Squares	Degrees of Freedom	Mean Squares	F	P
Total	6,674·44	15			
Between doses	2,779·69	3	926·56	24·6	<0·01
,, rows	2,397·69	3	799·23	20·4	<0·01
,, groups	1,260·69	3	420·23	10·7	<0·05
Error (residual)	236·99	6	39·5		
Linearity	2,773·18	1	2,773·18	70·5	<0·01
Quadratic	0·06	1	0·06		
Residual	6·51	1	6·51		

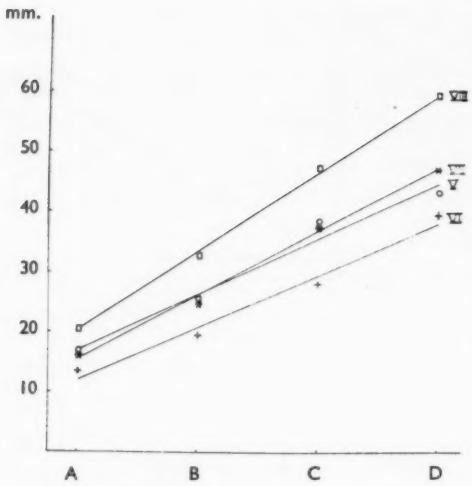

FIG. 4.—Tests of linearity. Theoretical lines calculated upon the means of the responses. Each line corresponds to a Latin square (Table VI).

" responses on levels of desensitization or tachyphylaxis."

When several doses are applied according to the schedule of the Latin square utilized for the test of linearity, the means of " Rows " can be employed for the calculation of Δ, since in this design the first " Row " (T_0) is composed of the responses to a first addition of each one of the four doses of anaphylatoxin, the second " Row " (T_1) of the responses following *one* previous addition of anaphylatoxin and so forth. It is clear that *levels of desensitization*, or the number of previous additions of anaphylatoxin, refer to the index of the totals for " Rows." The square given in Table IV can be taken as a model. If the regression " means of rows on levels of desensitization " are calculated by the least square method, Δ can be calculated as the quotient of the slope (b') of the line by the maximum ordinate (Y_0). This definition of Δ agrees well with the definition previously given. (In the following discussion, b' will always indicate the slope of the regression " responses on levels of desensitization," since b has been already utilized to indicate the slope of the regression " responses upon log dose of anaphylatoxin ").

In a separate series of experiments, we have attempted a direct estimate of this coefficient of desensitization (Δ). For this purpose, the main argument of the previous design has been inverted and four doses of anaphylatoxin (the same as those employed in the sequences of Table VI) were added repeatedly to four pieces of ileum. Here, variation due to doses was confounded with group variation (inter-intestines) and therefore discarded in the corresponding analysis of variance. The sequences employed were the following:

Groups:	I	II	III	IV	Totals T_R	Means
Rows	A_0	B_0	C_0	D_0	T_0	Y_0
	A_1	B_1	C_1	D_1	T_1	Y_1
	A_2	B_2	C_2	D_2	T_2	Y_2
	A_3	B_3	C_3	D_3	T_3	Y_3

A typical set of responses is presented in Fig. 5. The totals of " Rows " represent the responses to the four doses of anaphylatoxin in different *levels of desensitization*. It is obvious that the first total T_0 is composed of the full responses to the four doses in a level (0) of desensitization; the following totals T_1, T_2, T_3 . . . correspond to the responses in 1, 2, 3 . . . levels of desensitization, due to the tachyphylactic effect. It is easy to conclude that the best method for the estimation of Δ, consists in the calculation of the regression lines, using as dependent variable the means Y_0, Y_1, Y_2 . . . corresponding to the totals and as independent

TABLE VI

SUMMARY OF ANALYSIS OF 8 SQUARES DESIGNED FOR TEST OF LINEARITY

Doses in ml.: A, 0·02; B, 0·03; C, 0·045; D, 0·0675

Expt.	Square	Variance of Error (s^2)	Stand. Deviation (s)	Slope (b)	Ratio $(s\,b) = \lambda$	Theoretical Equations ($X = \text{Log}_{10}$ dose)	Mean Square Variances Corresponding to " Between Doses "		
							Linear	Quadrat.	Res.
1	I	57·72	7·60	59·8	0·13	$Y = 1{\cdot}16 + 59{\cdot}8X$	2,111·5	189·0	85·12
	II	39·40	6·28	66·9	0·09	$Y = -1{\cdot}37 + 66{\cdot}9X$	2,773·1	0·06	6·51
	III	96·75	9·83	55·1	0·18	$Y = -12{\cdot}4 + 55{\cdot}1X$	1,881·8	0·25	36·45
	IV	48·91	6·99	75·0	0·09	$Y = -2{\cdot}37 + 75{\cdot}0X$	3,406·5	30·25	92·00
2	I	19·00	4·30	54·1	0·08	$Y = 1{\cdot}00 + 54{\cdot}0X$	1,795·5	10·56	25·93
	II	74·33	8·62	50·6	0·17	$Y = -3{\cdot}07 + 50{\cdot}6X$	1,584·2	30·25	0·55
	III	63·17	7·96	61·4	0·13	$Y = -2{\cdot}80 + 61{\cdot}4X$	2,332·8	1·00	9·20
	IV	29·17	5·40	76·2	0·07	$Y = -2{\cdot}36 + 76{\cdot}2X$	3,604·6	0·62	0·88

Homogeneity of variances: $\chi^2 = 4{\cdot}134$. Degrees of freedom, 7. $0{\cdot}70 < P < 0{\cdot}80$.

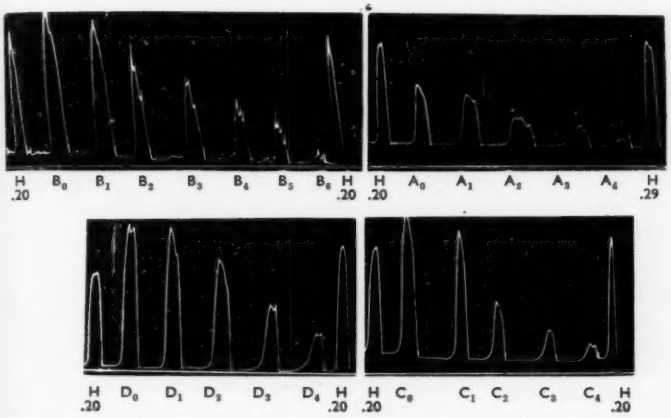

FIG. 5.—Design to calculate the regression "responses on levels of desensitization." To each piece of ileum the same dose of anaphylatoxin was repeatedly applied. H, 0.2 ml. histamine solution 1 : 2 millions.

variate the different *levels of desensitization* (0, 1, 2, 3 . . .).

In 3 different experiments so designed, the following totals were obtained:

Levels of desensitization:	0	1	2	3
Square No.				
I	145·5	110·5	82·0	57·0
II	184·5	142·0	101·0	69·0
III	160·5	142·0	103·0	59·0

The simplest inspection of these data shows that there is a regression of totals of "Rows" (or their corresponding means) upon the levels of tachyphylaxis. If the means are taken and the lines calculated by the least square method, the slopes will give a quantitative measure of the tachyphylactic effect, since the coefficient of desensitization (Δ) indicates the slope of the regression line (b') divided by the maximum ordinate (Y_0) of the theoretical line: $\Delta = b'/Y_0$.

The analysis of variance shows that the "linear" component of the variance due to the tachyphylactic effect is highly significant, and that the combined "Quadratic Residual" component is trivial and non-significant (Table VII). In Fig. 6, the means corresponding to the totals for "Rows" (T_R) are plotted against the four "levels of desensitization," showing the good fit of the experimental data with the regression lines. Table VII shows also the values of Δ, deduced from the experimental data. Its constancy is good evidence that the tachyphylaxis follows a regular course in different pieces of ileum taken from different guinea-pigs.

The totals of "Rows" in the experiments of Table IV have also been used to calculate Δ, by the method described above (Table VIII). Here, too, the regression "responses upon levels of desensitization" is linear, since deviations from

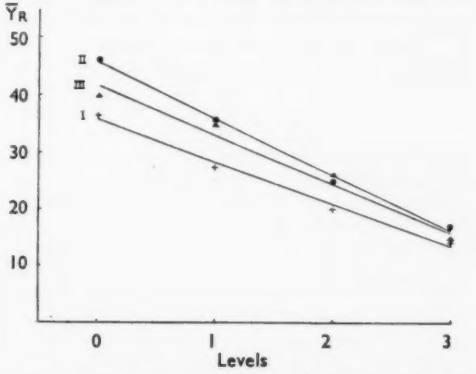

FIG. 6.—Regression lines "means of rows on levels of desensitization." Ordinates, means of "rows." Abscissae, number of previous additions of the same dose of anaphylatoxin. Each line represents the average of four doses.

TABLE VII

REGRESSION LINES AND ANALYSIS OF VARIANCE OF THE EXPERIMENTS DESIGNED TO ESTIMATE THE COEFFICIENT OF DESENSITIZATION

Expt.	Theoretical Lines	Coeff. Desens. (Δ)	Sum of Squares "Between *Levels*"			Error Variance
			Linear	Quadratic	Residual	
1	$Y = 35·6 - 7·4X$	0·20	1,065·80*	5·64†	0·78†	31·59
2	$Y = 45·5 - 9·7X$	0·21	1,876·95*	6·89†	0·66†	18·32
3	$Y = 42·0 - 8·6X$	0·20	1,474·90*	40·64†	2·96†	33·08

*, Highly significant; †, non-significant.

TABLE VIII

DATA FROM EXPT. 2, TABLE IV

Regressions of "means of rows on levels of desensitization" and coefficient of desensitization

Expt.	Theoretical Lines	Coeff. Desens. (Δ)	Sum of Squares "Between *Levels*"			Error Variance
			Linear	Quadratic	Residual	
1	$Y=48{\cdot}8-11{\cdot}5X$	0·23	2,663·17*	51·56†	62·83†	19·00
2	$Y=43{\cdot}7-12{\cdot}7X$	0·28	2,953·45*	196·00†	7·00†	74·33
3	$Y=49{\cdot}8-11{\cdot}9X$	0·24	2,856·05*	36·00†	24·95†	63·17
4	$Y=61{\cdot}2-13{\cdot}6X$	0·22	3,712·81*	38·06†	47·13†	29·17

*, Highly significant; †, non-significant.

linearity were non-significant when compared with the Error variance.

Correction for Group Differences and for Δ.—Now, if we take $\Delta=0.22$ for Square IV, Table VIII, and apply a correction to each single response according to its place in the sequence of treatments, a corrected square can be obtained. Fig. 7 gives a graphical representation of this correction operating upon the individual columns of the square. In (a) the experimental data are presented without any correction; in (b) the individual data have been corrected for the tachyphylactic effect, considering $\Delta=0.22$; and in (c) a further correction for the "Group" differences has been applied. It is apparent that the initial scatter of the data about the means becomes corrected, giving an even distribution around the theoretical line.

DISCUSSION

The analysis of variance of the assays presented in this paper shows that the so-called (2 + 2) design can be successfully applied for potency estimations of a material inducing strong tachyphylaxis. The variation from this effect, being due to a systematic source, could be confounded with the order of additions in the common Latin square design, and its sum of squares eliminated from the experimental error, thus increasing the precision of the assay several-fold. If one recalculates the variance of the Error, in the experiment analysed in Table II, including the sum of squares corresponding to "Rows," with 9 degrees of freedom, the corresponding variance will be 296.9 and the standard deviation of a single response will be 17.2 instead of 6.67—a considerable decrease of from 6- to 7-fold of the "information" given by the assay. Deviations from parallelism in all assays studied only once attained the probability level of 5%, and in most assays the sum of squares corresponding to "Parallelism" was of the same, or of a lower, order of magnitude as the Error variance. On a special sequence of assays designed to test linearity, using 2 series of 4 squares each, there was no significant departure from linearity in any single square; only once (Square I, Table VI) the variance ratio (F) corresponding to the "Quadratic" term went up to 189.0/57.7=3.3, still non-significant at the 5% level of probability.

In a total of 13 squares (Table III), the ratio b/s_b was above 10.0 in five, above 7.5 in nine; and only in four squares (Square IV, Expt. 1; Square II, Expt. 2; and Squares I and II, Expt. 3) was this ratio too low for the application of the simplified formula for the calculation of the Error of M (s_M). Therefore, in more than 2/3 of the assays, the application of the simplified formula for s_M was well warranted by the intrinsic qualities of the assays. Considerations derived from the values of $\lambda=s/b$, the "standard deviation in terms of log dose," permit an assessment of the efficiency of each response. In this respect, the present assay is less

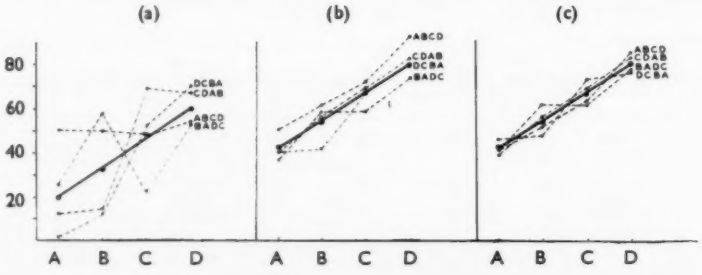

FIG. 7.—Corrections applied to the data of one square utilized in the test of linearity. (a) Without correction; (b) corrected for $\Delta=0.22$; and (c) corrected for Δ and for Group differences.

efficient than similar assays performed with histamine or bradykinin, for instance, in which λ tends to a value below 0.050. However, the precision of the assay (measured as the reciprocal of the Error variance) is about a quarter of that with histamine or bradykinin, but still above the precision of many biological assays with insulin or vitamins (for comparison, see Bliss and Cattell, 1943). Obviously, the precision of a method will depend on the care with which the biological preparation is handled, on accurate measurements of volumes, and on registration of the responses in each replication. This last source of variation is increased when a fresh preparation has to be set up for each new replication of the basic sequence of treatments. The histamine and bradykinin assays can be replicated as many times as necessary upon a single piece of intestine. In an assay involving a strong tachyphylactic effect we are fortunate to be able to assay a whole sequence of treatments (4) constituting a " Group " on the same piece of intestine, as in the present experiments. Then, by confounding inter-intestine variation with variation " Between groups," the precision is considerably increased. However, some inherent variation still remained and it could not be eliminated by the design adopted.

The precision obtained with a single square can be satisfactory for many purposes. If we take the antilog of $s_{\bar{M}}=0.050$ for the first assay of Table III, equal to 1.12, it would indicate a standard deviation of $\pm 12\%$ in the estimation of a single ratio of potency; if we take the antilog of $s_{\bar{M}}=0.031$, corresponding to the average $\bar{M}$ of the 5 squares (80 responses), the standard deviation will not exceed $\pm 7.4\%$. In general, if we assume that $\lambda=0.10$, we can calculate how many treatments (N) would be necessary to keep $s_{\bar{M}}$ below, say, 0.030. According to Bliss (1952):

$$N=\frac{4\lambda^2}{s^2_{\bar{M}}}$$

Substituting the data for the letters in this formula, N would be about 40, and if we make an allowance for the variation of λ, and taking $s_\lambda=0.03$, the safest number would be around 96 treatments or about 6 squares. Such a precision, bringing down the standard deviation to $\pm 7.2\%$, will probably not be necessary for a common assay of estimating the potency ratio of two preparations of anaphylatoxin. If one is satisfied with a standard deviation of $\pm 10\%$, corresponding to a $s_{\bar{M}}=0.042$, two squares would be theoretically enough.

Of interest for the study of the intimate mechanism of the tachyphylactic phenomenon is the possibility of estimating Δ, coefficient of desensitization. It was defined as the quotient of the slope of the line by the first response (Y_0) to a single dose of anaphylatoxin. In fact, we are dealing here with effects of " intrinsic " histamine (Dale, 1948), since the responses obtained by repeating the additions of the same dose of anaphylatoxin upon the same piece of ileum are due to successive levels of released histamine. What we have called the regression of " rows on levels of desensitization," or simply " responses on previous additions of anaphylatoxin," actually is a regression of " responses on *doses* of intrinsic histamine," and the fact that it is negative only indicates that the amounts of released histamine are smaller and smaller as tachyphylaxis sets in.

It might be useful to speculate further on the intimate nature of the regression " responses on levels of desensitization." The fact that it is linear, as indicated by the analysis of variance, shows that the process of release is of such a nature that the responses to the successive amounts of the released metabolite are linearly decreased following each new addition of the releasing agent. On this basis, it would be interesting to deduce an arbitrary *scaling* for doses of *intrinsic* histamine released at successive steps of the tachyphylactic effect. The simplest assumption concerning the mechanism of this phenomenon is that the " available " stock of the metabolite becomes more and more depleted and that any further dose of anaphylatoxin will release a certain percentage of the residual " available " stock. This assumption is a natural one, and warranted by previous experiments dealing, for instance, with the release of histamine from the rat's diaphragm by successive contacts with the same concentration of (+)-tubocurarine (Rocha e Silva and Schild, 1949). If it is so, the law regulating this intrinsic phenomenon could be expressed by the exponential

$$D=Ce^{-ax}$$

where x will be 0 or any positive integer from 1 to 3 or 4, until the amount of released histamine becomes too small to produce a measurable contraction, and a and C are constants. Now, if we take the natural logarithms of the doses of released histamine (D), as an expression of the responses obtained at the different levels of desensitization, we will have the equation:

$$Y=k\log_e(Ce^{-ax})=k\log_e C-akx$$

where k is a new constant, and Y the response in mm.

This equation must correspond to the linear relation between the responses and the number of previous additions of the same dose of anaphylatoxin, and therefore could be identified with the

experimental equations of Table VII, calculated by the method of least squares. It is clear that ak can be identified with the slope b' of these lines, and since $\Delta = b'/Y_0$ and $Y_0 = k \log_e C = (b'/a) \log_e C$ we can conclude that $\Delta = a/\log_e C$.

By simple substitution, the above equation can be presented in the form:

$$Y = b'/\Delta - b'x$$

If we take, for instance, the experimental equation for Square II (Table VII)

$$Y = 45.5 - 9.7x$$

it can be compared with the theoretical one deduced above, by making $b' = 9.7$ and $\Delta = 0.21$:

$$Y = 46.0 - 9.7x$$

showing a most excellent agreement between theory and experiment.

Summary

1. A (2+2) assay designed as a 4×4 Latin square is described to estimate a ratio of potency between two solutions (unknown and standard) of a material producing tachyphylaxis when assayed upon the guinea-pig ileum. The material utilized—rat's plasma activated with agar (anaphylatoxin)—has been shown previously to stimulate the guinea-pig ileum by release of histamine.

2. To test linearity of the log dose response curve, the same Latin square design was utilized. Deviations from parallelism and from linearity were almost always trivial and non-significant.

3. The theoretical model adopted, by assuming that each dose affects the responses of any subsequent addition of anaphylatoxin by a certain residual value, introduced the idea of a coefficient of desensitization Δ. The method for its calculation is described.

4. The negative regression " responses on number of previous additions of anaphylatoxin " indicating a linear downward trend of the responses to the same dose of anaphylatoxin has been understood as a linear regression of " responses on doses of *intrinsic* histamine." An arbitrary scaling of doses, $D = Ce^{-ax}$, of intrinsic histamine has been derived from considerations concerning the mechanism of release. By taking the natural logarithms of the doses of intrinsic histamine, as an expression of the responses $Y = \log_e D$, the negative linear regression so obtained could be identified with the regression of " responses on number of previous additions of anaphylatoxin."

The authors are grateful for Professor W. L. Stevens's statistical comments and criticism. The work was performed during the tenure by one of us (A. M. R.) of a fellowship of the Brazilian National Research Council.

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THE EFFECT OF ACETAZOLEAMIDE, AN INHIBITOR OF CARBONIC ANHYDRASE, ON GASTRIC SECRETION

BY

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Sulphanilamide has a diuretic effect (Southworth, 1937; Strauss and Southworth, 1938) which is thought to be due to inhibition of the enzyme carbonic anhydrase, which catalyses the reversible reaction

$$CO_2 + H_2O \rightleftharpoons H_2CO_3$$

Acetazoleamide (2-acetylamino-1, 3, 4-thiadiazole-5-sulphonamide, "Diamox"), a derivative of sulphanilamide, is a more powerful inhibitor of carbonic anhydrase than sulphanilamide.

Davenport and Fisher (1938) demonstrated that carbonic anhydrase is present in large amounts in the gastric mucosa, and later (Davenport, 1939, 1940) that most of this enzyme in the stomach is present in the oxyntic cells. Further investigations have sought to prove that the enzyme plays an important part in gastric hydrochloric acid secretion. Janowitz, Colcher, and Hollander (1952) showed that histamine-induced secretion in dogs could be completely suppressed by acetazoleamide. The mechanism of gastric HCl secretion has been reviewed recently by Gray (1942), Davies (1951), Texter and Barborka (1955).

In view of the fundamental role played by carbonic anhydrase in gastric acid secretion it was suggested that an inhibitor of the enzyme might be of use in the treatment of the hyperacidity associated with peptic ulcers. The present study was undertaken to assess the effects of acetazoleamide on gastric acid secretion. Parallel studies on urinary volume, electrolyte excretion, and urinary *p*H were performed to make sure that the drug had reached an effective level.

METHODS

The drug was administered to twelve healthy normal adults isolated in a small hospital ward. They were kept on a fixed weighed diet for three days, additional fluid intake being restricted to 1,500 ml. On the first day they accustomed themselves to the diet, the second was the control day and the third the experimental day. The subjects were divided into two groups of 6, one of which received 250 mg. and the second 500 mg. acetazoleamide on the test day.

The effect of acetazoleamide on gastric secretion was compared with that of atropine, a known inhibitor of acid secretion. Two mg. of atropine was given subcutaneously an hour before gastric analysis to another group of six subjects treated under similar conditions.

If a standard volume of a non-absorbable dye such as phenol red is injected into and withdrawn from the stomach at a fixed interval, it is possible to calculate the volume of pyloric evacuation and the rate of gastric secretion (Brooks, Erskine, Gephart, Swain, and Moore, 1950; Gill and Jessup, 1950) on the assumption that the rate of pyloric evacuation is constant. Although the method is open to the errors inherent in this assumption, it provides a quantitative assessment of the volume of gastric secretion which cannot be obtained with fractional meal techniques.

The following investigations were performed on control and test days:

1. The phenol red method of gastric analysis of Brooks *et al.* (1950) was used. The procedure for gastric analysis was modified from Gill and Jessup (1950). Subjects were fasted for twelve hours. A Ryle's tube was passed and the resting juice aspirated. A solution of phenol red (100 ml. of 1.2 mg.%) warmed to 37° C. was injected into the stomach by way of the Ryle's tube; ten minutes later the gastric contents were completely aspirated. A further 100 ml. of phenol red solution was immediately injected and the procedure repeated for five 10-min. periods. The volumes of aspirates were recorded and the concentration of phenol red estimated in an EEL absorptiometer against a control sample of the injected solution (to allow for any concentration during warming to 37° C.).

2. The volume and specific gravity of the urine passed during the two 24-hr. periods covering the control and experimental days were measured.

3. A urine concentration test was made involving the collection of three urine specimens passed at hourly intervals in the early morning before gastric analysis and the determination of their volumes, specific gravity, and *p*H.

4. The 24-hour sodium and potassium excretion was measured on control and test days.

5. Plasma chlorides were determined by the method of Schales and Schales.

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Results

The rates of total gastric secretion, hydrochloric acid production and pyloric evacuation were calculated and the results for each of the three groups are presented in Table I. The statistical significance of differences between results on control and test days was calculated by students "paired" *t* test.

TABLE I

THE EFFECT OF ACETAZOLEAMIDE ("DIAMOX") ON VOLUMES OF PYLORIC EVACUATE, GASTRIC SECRETION AND FREE ACID SECRETION IN GROUPS OF 6 NORMAL ADULTS, CONTRASTED WITH THE EFFECT OF ATROPINE

Drug and Dose	Subject	Pyloric Evacuate (ml./min.)		Gastric Secretion (ml./min.)		Free Acid (mMol./min.)	
		Before	After	Before	After	Before	After
Acetazoleamide 500 mg. by mouth	A	10	8·4	1·8	1·3	0·005	0·007
	B	3·2	8·0	1·6	0 66	0·029	0·013
	C	3·4	3·9	0·56	1·8	0·001	0·011
	D	9·7	2·0	1·6	0·68	0·042	0·008
	E	7·5	4·6	2·4	2·5	0·028	0·008
	F	5·6	5·8	3·1	0·2	0·005	0
	Mean	6·6	5·4	1·8	1·2	0·018	0·008
	P	50%		30%		<20%	
Acetazoleamide 250 mg. by mouth	G	12	7·6	2·6	1·1	0·048	0·035
	H	7·8	0·78	6·0	2·3	0·092	0·012
	J	11	7·5	4·1	4·1	0·045	0·013
	K	8·4	3·5	4·0	2·8	0·110	0·047
	L	0·5	5·0	1·9	1·6	0·017	0·014
	M	9·1	9·9	1·6	0·65	0·019	0·071
	Mean	8·3	5·7	3·4	2·1	0·055	0·032
	P	20%		5–10%		30%	
Atropine 2 mg. s.c.	N	10	2·3	2·7	0·8	0·019	0
	O	11	2·1	3·6	0·6	0·062	0·003
	P	6·8	5·0	1·9	0·4	0·034	0
	Q	11	2·1	4·6	0·86	0·12	0
	R	11	4·1	6·0	1·1	0	0
	S	8·4	8·7	3·2	1·0	0·042	0
	Mean	9·7	4·1	3·7	0·72	0·046	0
	P	<25%		<1%		<5%	

Gastric Function.—The rate of gastric secretion, the production of acid and the rate of pyloric evacuation were all slightly diminished after the administration of acetazoleamide, but the decrease was small and not statistically significant. Furthermore, a dose of 500 mg. acetazoleamide produced no greater effect than a dose of 250 mg. By contrast 2 mg. atropine produced a significant diminution of gastric secretion and motility.

Urine Output and Urinary Electrolytes.—In all instances the total volumes of the three morning specimens and the volumes of the 24-hour output were greater after the administration of the drug. The volumes of the 24-hour specimens increased on an average by a factor of 1.86 on 500 mg. and 1.7 on 250 mg. acetazoleamide. The concentration of the urine was reduced and the urine became alkaline in all cases. The effect of acetazoleamide on the excretion of sodium and potassium is shown in Figs. 1, 2, and 3.

The increase in sodium excretion over the 24-hour period corresponded closely with the increase in 24-hour volume. All subjects likewise showed an increased potassium excretion which was greater on the experimental day by a factor of 1.7 on 500 mg. and 1.6 on 250 mg. acetazoleamide. The plasma chlorides showed no significant change.

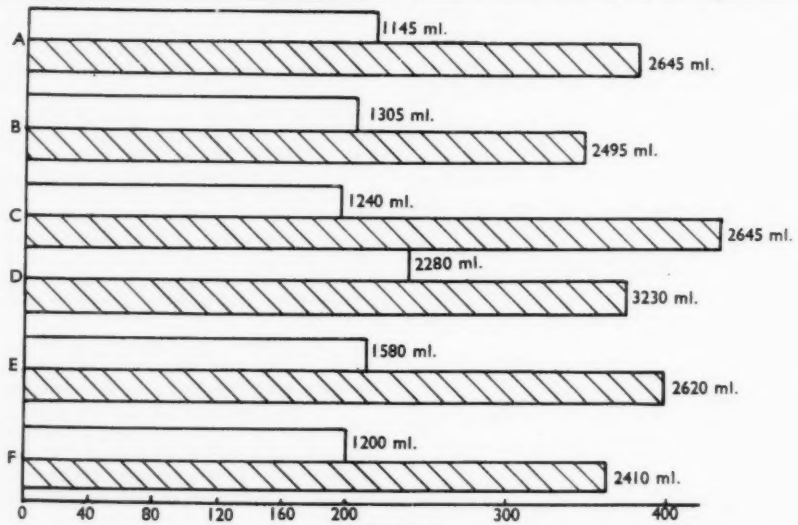

FIG. 1.—The effect of oral administration of 500 mg. of acetazoleamide on the total daily urinary excretion of sodium in 6 healthy adults. Plain rectangles before, and hatched rectangles after, acetazoleamide.

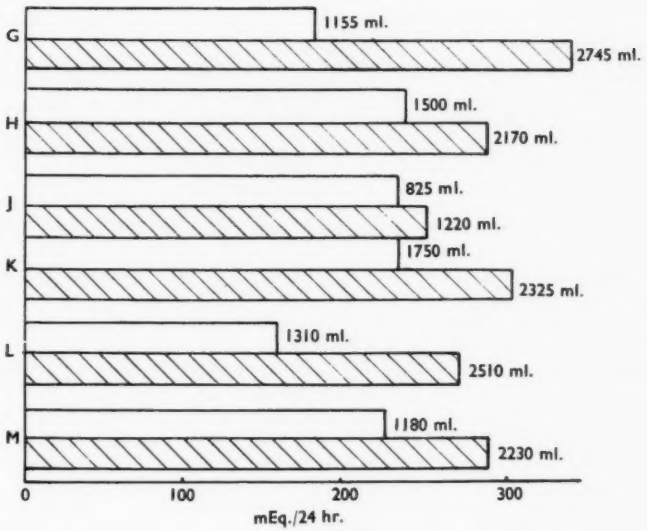

FIG. 2.—The effect of oral administration of 250 mg. of acetazoleamide on the total daily urinary excretion of sodium in 6 healthy adults. Plain rectangles before, and hatched rectangles after, acetazoleamide.

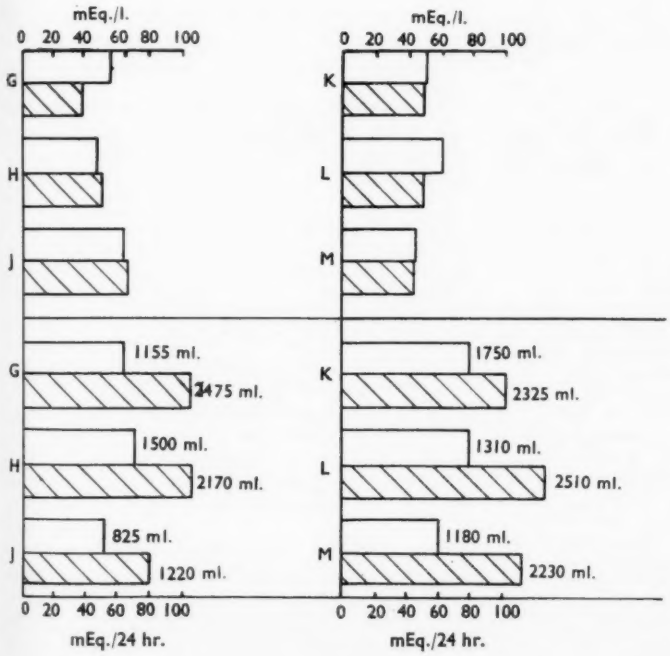

FIG. 3.—The effect of oral administration of 250 mg. of acetazoleamide on urinary potassium excretion in 6 healthy adults. Plain rectangles before, and hatched rectangles after, acetazoleamide.

Discussion

Acetazoleamide in the usual therapeutic doses did not abolish gastric hydrochloric acid secretion. Counihan, Evans, and Milne (1954) consider that renal carbonic anhydrase activity is not completely suppressed by therapeutic doses of acetazoleamide. Failure to reduce gastric function significantly is probably due to the same cause, as the concentration of carbonic anhydrase in the oxyntic cells is relatively high (Davies and Edelman, 1951).

The present experiment is the first in which the effects of acetazoleamide have been studied in a relatively large group of normal adults. The use of the drug as an oral diuretic in congestive heart failure and emphysema has been the subject of recent clinical reports (Belsky, 1953; Ruskin, 1955). The paper by Counihan, Evans, and Milne (1954) described the action in two normal adults and a group of patients. In our twelve subjects we have confirmed the marked diuretic action of acetazoleamide, and parallel effects on soduim and potassium excretion, in the first twenty-four hours after administration.

Acetazoleamide does not appear to possess any advantages over conventional methods for the treatment of hyperacidity; its other important physiological effects, such as the production of systemic acidosis, would make prolonged therapy of such gastric disorders unnecessarily hazardous.

Summary

1. The effect of acetazoleamide ("Diamox"), an inhibitor of carbonic anhydrase, on gastric secretion has been studied in twelve healthy (normal) adults on a similar diet under hospital conditions.

2. In therapeutic doses acetazoleamide does not appear to have a marked effect on gastric hydrochloric acid secretion; it has a much less significant effect on total gastric secretion than atropine. Marked diuresis, increased sodium and potassium excretion, and alkalinization of the urine, were observed in all subjects, in the first twenty-four hours after administration.

Permission to publish this work has been granted by the Ministry of Supply. Sodium and potassium determinations were kindly performed by Dr. T. Shaw.

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THE PHARMACOLOGY OF BENZOYLCHOLINE DERIVATIVES AND THE NATURE OF CARBONYL RECEPTORS

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Little is known of the function of the carbonyl group in the pharmacological action of choline esters; hitherto the only satisfactory method of study has been by observing the effects of altering the acetylcholine molecule so that other parts of the molecule bear different spacial relationships to the carbonyl group (Welsh and Taub, 1951; Ing, Kordik, and Tudor Williams, 1952). Another method of study is presented in this paper; it has already been used to obtain information on the interaction of cholinesterase and the carbonyl group of benzoylcholine (Ormerod, 1953).

A series of derivatives of benzoylcholine was synthesized having substituents in the benzene ring such that the reactivity of the carbonyl group was affected by the electron attraction or repulsion caused by the substituents: the degree of this attraction or repulsion (mesomeric effect) is given for each substituent by the substituent constant S, where $S = \log K - \log Ko$, K being the dissociation constant of corresponding substituted benzoic acid and Ko the dissociation constant of unsubstituted benzoic acid (Hammett, 1940).

As a correlation had been demonstrated between the mesomeric effect and rate of enzyme hydrolysis —that is to say, the highly activated compounds such as *p*-chlorobenzoylcholine were hydrolysed rapidly, and "deactivated" compounds such as *p*-methoxybenzoylcholine slowly—and as evidence had been put forward to suggest that this correlation could be ascribed to the interaction of the carbonyl group with a complementary group on the enzyme surface, it was felt that a similar correlation found in any pharmacological action of this series would provide evidence of carbonyl receptors of pharmacological importance and give some indication of their chemical nature.

However, it was found that on some sites of action of the compounds no correlation between mesomeric effect and pharmacological action was shown; in these instances the work has not been pursued beyond the point where it became apparent that no such correlation existed. The "nicotine-like" actions of these compounds—both in raising the blood pressure of the atropine-treated cat and in stimulating the cat's superior cervical ganglion—did, however, show a degree of correlation with mesomeric effect, and the significance of this will be discussed later. In general, the methods used and sites of activity studied were those that would give satisfactory assays; the action on the guinea-pig's ileum—apparently an obvious choice—was avoided because of the complications described by Akcasu, Sinha, and West (1952).

MATERIALS AND METHODS

The series of substituted benzoylcholine derivatives had been synthesized and described previously (Ormerod, 1953). A further sample of the *p*-methyl derivative was prepared, to check the purity of the earlier sample, by reaction of *p*-toluoylchloride with ethylene chlorohydrin. In addition, *p*-chlorobenzoylcholine iodide was added to the series, synthesized by the standard method. Analysis by Drs. Weiler and Strauss. Found: C, 39.05; H, 4.5. $C_{12}H_{17}O_2ClI$ requires: C, 39.0; H, 4.5%. Substituent constant and rate of hydrolysis with horse-serum cholinesterase is given in Table I.

Action on the Neuromuscular Junction

Frog's rectus abdominis preparation was used as described by MacIntosh and Perry (1950): relaxation was slow and incomplete and the muscle had to be stretched between each addition of drug. Assays were based on the comparison of log dose-response line for the unknown with that of the standard benzoylcholine; two points on each line were determined, each representing the mean of four doses.

The phrenic nerve and diaphragm preparation of the rat was used as described by Chou (1947) with modification of West (1947). "Square wave" electrical stimuli excited the phrenic nerve at a rate of 8/min. at the minimum pulse width and voltage which produced maximal stimulation (usually 0.1 msec. and 4.5 v.). The solution contained: NaCl, 8.00; KCl, 0.20; $CaCl_2$, 0.20; NaH_2PO_4, 0.05; $NaHCO_3$, 1.00; dextrose, 1.00 g./l. The routine was otherwise as described by Burn (1950). Benzoylcholine was used as standard.

TABLE I

POTENCY OF BENZOYLCHOLINE DERIVATIVES (RELATIVE TO BENZOYLCHOLINE=1) ASSAYED ON FROG RECTUS ABDOMINIS AND RAT DIAPHRAGM PREPARATIONS

The dose limits for the standard benzoylcholine are given in parentheses. Also included are the substituent constant (S = log K − log Ko), and the rate of hydrolysis with serum cholinesterase (k_{30} = zero order velocity constant at 30° C.)

Derivative	Substituent Constant S	Rate of Hydrolysis k_{30}	Frog Rectus			Rat Diaphragm Blocking Action		
			Stimulation		Block			
			Without Eserine	With Eserine		Without Eserine	With Eserine 1 : 100,000	With Eserine 1 : 1,000,000
m-Nitro	+0·71	2·64	0·32 approx.	0·32 approx.	+	2·8	0·59	0·31
m-Chloro ..	+0·37	3·13	0·25 approx.	0·26 approx.	+	—	—	—
m-Fluoro ..	+0·34	2·70	—	—	—	—	—	—
p-Chloro	+0·23	1·94	0·23	—	—	1·2	0·24	0·81
p-Fluoro	+0·06	1·70	0·50	—	—	1·2	—	—
Unsubstituted ..	0·00	1·90	1·00 (0·1–0·5 mg.)	1·00 (0·1 and 0·23 mg.)	—	1·00 (4 and 6 mg.)	1·00 (1·9–2·0 mg.)	1·00 (0·5–1·2 mg.)
m-Methyl ..	−0·07	0·64	0·21	—	—	0·63	—	2·0
p-Methyl	−0·17	0·40	—	—	—	—	—	—
p-Methoxy ..	−0·27	0·1 approx.	0·23	—	—	2·5	0·71	1·6

Action on Blood Pressure

This was observed in cats anaesthetized with ether and chloralose (80 mg./kg.) and recorded at the carotid artery before and after 1 mg./kg. atropine sulphate. The potency of the derivatives in raising the blood pressure after atropine was estimated by giving each in a single high and a single low active dose; each of these doses was succeeded by an equipressor dose of the standard benzoylcholine. The log dose-response line was determined at the beginning of the experiment, and subsequent responses were compared with this curve. When the sensitivity of the preparation was found to have altered the results were rejected. The potency of each compound was determined from the horizontal distance of its log dose-response line from that of the standard. The combined result of experiments on two cats is shown in Table II: the result is approximate, since the log dose-response lines were not parallel, and the minimum of observations was made.

Action on Superior Cervical Ganglion

This was observed in cats by the method of Kibjakow (1933) and Feldberg and Gaddum (1934). The perfusion fluid contained: NaCl, 8.00; KCl, 0.20; $CaCl_2$, 0.20; $MgCl_2$, 0.01; NaH_2PO_4, 0.05; $NaHCO_3$, 1.00; dextrose, 1.00 g./l. and eserine sulphate 1/100,000 and was warmed by passing the tube up the animal's oesophagus (MacIntosh, 1949, unpublished); best results were obtained with perfusion pressures of 35–45 mm. Hg. Drugs were injected in solution (0.2 mg./ml.) through standardized micrometer syringes, one each for standard and unknown; these were inserted and clamped in position close to the carotid cannula. Two dose levels were used for each drug, and the injections were given in random order. Log dose-response lines were obtained by plotting the mean height of contraction in mm. against the logarithm of the dose. One ganglion was used for each compound assayed against benzoylcholine as standard. Observations were also made on the blocking action of these compounds, but no accurate assessment of their activity in this respect was possible.

A few observations were made with normal circulation through the ganglion; injections were made through a cannula inserted in the cut end of the carotid artery distal to the ganglion.

RESULTS

Tables I and II show the potencies of the derivatives of benzoylcholine relative to the parent compound. In most of the experiments eserine was used to inhibit hydrolysis, since if this precaution was omitted the type of correlation which appears could be attributed to hydrolysis of the compounds alone and not to differences in their pharmacological activity. This might be true of the experiments on the cat's blood pressure and on the superior cervical ganglion with normal circulation even in the presence of eserine, since it is impossible without giving toxic doses to be sure that all circulating esterase is inactivated: but it is unlikely that this mechanism is in fact operating, both because of the rapidity of response and because of the general agreement of results by these methods with those from perfusion of the superior cervical ganglion with a solution containing eserine to which there is no such objection.

The frog's rectus abdominis preparation shows no correlation between stimulant activity and mesomeric effect, but the two members with the most activating substituents, the *m*-nitro and the *m*-chloro derivatives, showed some blocking action so that the responses decreased rapidly and an accurate assay was impossible. The blocking action of these compounds to acetylcholine was negligible. Eserine had little effect on the action of these compounds on the frog rectus.

The neuromuscular-blocking action of these compounds on the rat diaphragm showed no correlation with mesomeric effect: eserine salicylate

TABLE II

NICOTINE-LIKE ACTIVITY (RELATIVE TO BENZOYLCHOLINE = 1) OF BENZOYLCHOLINE DERIVATIVES ASSAYED ON THE BLOOD PRESSURE AND ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

The dose limits for the standard benzoylcholine are given in parentheses. The figures in square brackets represent assays completed before the final method was evolved.

Derivative	Atropine-treated Cat's B.P.	Cat's Superior Cervical Ganglion		
		Arterial Injection Without Eserine	Perfusion With Eserine	Perfusion 95% Confidence Limits
m-Nitro ..	0·1 approx.	—	Nil	
m-Chloro ..	0·3 approx.	0·44	0·03 approx.	
m-Fluoro ..	—	—	0·218	0·171–0·226
p-Chloro ..	0·45	—	0·392 [0·53]	0·292–0·430
p-Fluoro ..	0·60	—	0·687 [0·56]	0·599–0·851
Unsubstituted	1·00 (0·5–2 mg.)	1·00 (50,100 and 200 μg.)	1·00 (2–3 μg.)	
m-Methyl ..	0·40	—	1·144	0·9119–1·490
			0·359 [0·4]	0·279–0·393
p-Methyl ..	0·6 approx.	—	0·339	0·302–0·373
p-Methoxy ..	2·16	2·8	1·812	1·281–1·960

1: 1,000,000 increased the blocking action of these compounds but reduced their potency relative to benzoylcholine; further reorientation of potencies occurred with eserine salicylate 1: 100,000. The meaning of these results is obscure.

The characteristic action of this series of compounds on the cat's blood pressure was a transient fall, usually described as a "muscarine-like" response: after atropine this was reversed, and a "nicotine-like" rise was obtained. In some animals only the rise in blood pressure was observed. The rise in pressure could be potentiated with eserine salicylate (1 mg./kg.), and an initial fall in pressure then preceded the rise, indicating a partial return of the muscarine type of response (Fig. 1). (This potentiation may have been produced by the inhibition of circulating cholinesterase, since it did not occur with the *p*-methoxy derivative which is hydrolysed by cholinesterase only to a negligible extent.) The rise in blood pressure was reduced to negligible size—as was that from an equipressor dose of nicotine—by hexamethonium bromide 10 mg./kg. The results shown in Table II were obtained after atropine and represent the nicotine-like action of these compounds: it was not possible in these experiments to make an accurate assessment of the potency of these compounds in producing muscarine-like activity, but it appeared to be similar—that is to say, a compound that gave a large rise in pressure after atropine could give a similarly large fall in the untreated animal. For these experiments 5 animals were used.

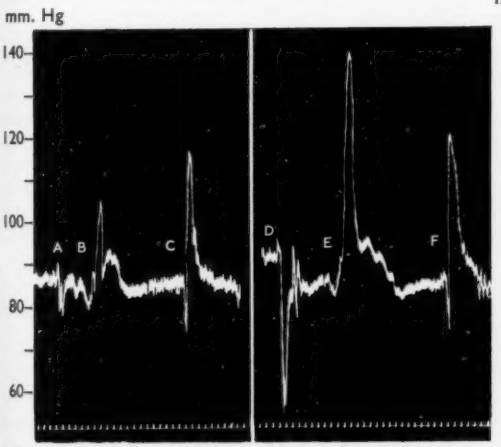


FIG. 1.—Cat, ether, chloralose. Arterial blood-pressure tracing to show "muscarine-like" and "nicotine-like" responses produced by 1 mg. benzoylcholine and 1 mg. *p*-methoxybenzoylcholine. A, benzoylcholine alone; B, benzoylcholine after atropine sulphate 1 mg./kg. C, benzoylcholine after atropine and eserine salicylate 1 mg./kg. D, p-methoxybenzoylcholine alone; E, p-methoxybenzoylcholine after atropine; F, p-methoxybenzoylcholine after atropine and eserine. Time in 10 sec.

Few experiments were performed on close arterial injection of the drug into the superior cervical ganglion, since this is open to the same objection as experiments on the blood pressure—namely, that the results might be due to differences in rapidity of hydrolysis and not to differences in pharmacological action.

The main effort of this work was therefore placed in an attempt to obtain an accurate assay of activity in the eserine-treated perfused superior cervical ganglion. The results, with their confidence limits, are shown in Table II. Ganglion-blocking activity, which could not be assessed accurately, was considered only in relation to stimulation with benzoylcholine and its possible effect on the assay of these compounds. (The blocking activity of these compounds also affected stimulation of the ganglion by excitation of the afferent nerve and by injection of acetylcholine, but no detailed study of blocking action was made in relation to these methods of stimulation.) *m*-Nitrobenzoylcholine produced block at a dose which did not stimulate; *m*-chlorobenzoylcholine produced block at the minimum stimulatory dose; with the other compounds block was produced only by a dose above that necessary to produce maximal stimulation. With most compounds there was a reasonable difference between the dose producing maximal stimulation and that which produced block, but this was not

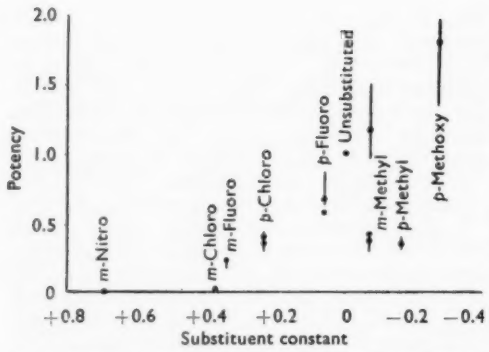


FIG. 2.—Potency of benzoylcholine derivatives (relative to benzoylcholine=1) as ganglion-stimulating agents, plotted against substituent constant (measuring electron drift in the benzene ring). Nicotine-like activity decreases with electron attractive power of substituent.

so with the *m*-methyl and *p*-methyl derivatives—in fact several assays with *p*-methylbenzoylcholine had to be rejected before one which was not affected by ganglion block was obtained.

The stimulating activity of these compounds on the superior cervical ganglion of the cat is shown, plotted against the substituent constant, in Fig. 2. As a definite correlation is shown between activity and mesomeric effect of substituents in the benzene ring, it can be postulated with reasonable certainty that carbonyl receptors exist, and that they are of importance in the nicotine-like stimulant activity of some choline esters on ganglia. *p*-Methylbenzoylcholine and one assay of *m*-methylbenzoylcholine lie, however, outside this correlation.

DISCUSSION

The deviation of *m*-methylbenzoylcholine and *p*-methylbenzoylcholine from the general scheme shown in Fig. 2 is not perhaps surprising in view of their greater blocking activity, yet I do not consider that the low values that they gave on assay were due solely to their blocking action, although this property serves to show that the pharmacological action of these compounds differs in some degree from those of other members of the series. This is also shown by their more prolonged action and irregularity of potency (the two assays of *m*-methylbenzoylcholine were both satisfactory as assays but gave widely different values). The possibility was considered that they might contain impurities, and it seemed likely that in the method of synthesis used the methyl groups of *m*- and *p*-toluic acids could have been oxidized to carboxylic acid groups, giving the *bis*-choline esters of, respectively, phthalic and *tere*-phthalic acids, configurations which might be expected to block ganglia; yet this possibility was eliminated both by study of analyses (see Ormerod, 1953) and by preparation of the *p*-methyl derivative by another synthetic route mentioned earlier in this paper. The sample so obtained showed similar potency and blocking action.

Ganglion stimulation has for long been associated with two types of choline derivate—the esters, both of aliphatic and aromatic acids, and the phenolic ethers. Quaternary ammonium compounds of similar structure but with a ketone group have recently been found active. It has been difficult hitherto to see how a single chemical grouping on the active surface of the ganglion can account for the activity of these three different chemical types.

There are few satisfactory references to the relative potency of choline esters, since most experiments have been performed on the whole animal and it is not always easy to estimate the part played by ester hydrolysis. It is clear, however, that acetylhomocholine (γ-acetoxypropyltrimethylammonium) and acetylformocholine (acetoxymethyltrimethylammonium) are active (Hunt and Taveau, 1911; Hunt and Renshaw, 1925) although less active than acetylcholine. Benzoylhomocholine is also active (Hunt and Taveau, 1906), possibly as active as benzoylcholine; benzoylformocholine does not seem to have been prepared. Of the ketones with 5 carbon chain studied by Welsh and Taub (1951), and by Ing, Kordik, and Tudor Williams (1952), 4-ketoamyltrimethylammonium, 3-ketoamyltrimethylammonium and 2-ketoamyltrimethylammonium were all active stimulators; however, among the phenolic ethers, β-phenoxyethyltrimethylammonium (the choline derivative) is highly active, whereas γ-phen-

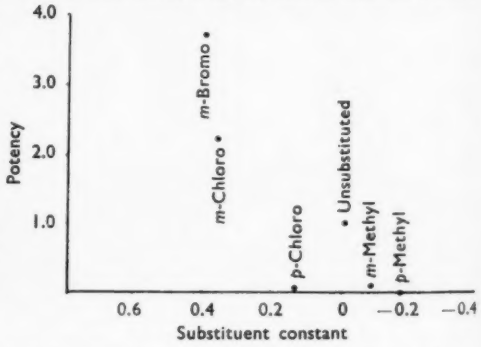


FIG. 3.—Potency of choline phenylether derivatives (relative to β-phenoxyethyltrimethylammonium=1) as pressor agents in atropine-treated cats (results of Hey, 1952) plotted against substituent constant. Nicotine-like activity increases with electron attractive power of substituent.

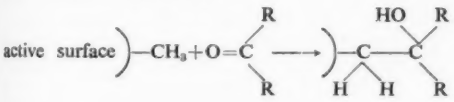
oxypropyltrimethylammonium (derivative of homocholine) is quite inactive (Hunt and Renshaw, 1929): so far as the evidence goes, there seems to be greater structural specificity in the ethers than in the carbonyl compounds.

A striking difference in the mechanism of action of choline esters and ethers is shown by comparing the results given in this paper with those of Hey (1952) (Fig. 3). Hey studied the activity of a series of substituted derivatives of the phenolic ether of choline, β-phenoxyethyltrimethylammonium bromide, and found that the activity in raising the blood pressure of the atropine-treated cat was *increased* by the insertion of electron attractive groups. As the activity of benzoylcholine derivatives tends to be inhibited by this type of substitution, it is probable that there are two different types of chemical reaction operating. With ethers the active surface of the ganglion appears to act as an " electrophilic " reagent and with esters as a " nucleophilic " reagent.

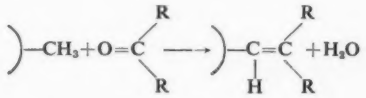
Proposed Hypothetical Carbonyl and Ether Receptor

It may be of interest at this point to put forward a possible mechanism by which the active surface of the ganglion could react both with carbonyl and ether groups. It is of course possible that two groups can coexist on the surface; but it is not a necessary assumption, since a single reagent can react with electron-rich or electron-deficient groups if it is capable of behaving as a weak acid. Such a group—put forward as an example, rather than as an hypothesis, since another weak acid would probably do as well—would be the activated methyl group as in the Knövenagel type of reaction.

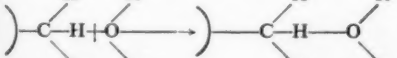
(*a*) for carbonyl group



OR



(*b*) for ether group



In (*a*) the ganglion surface is acting as a nucleophilic reagent and would condense less readily with an electron deficiency in the carbonyl group created by an electron attractive group in the benzene ring. In (*b*) the " hydrogen bond " is invoked; this is at its strongest when the hydrogen atom is acidic as in the activated methyl group and when the oxygen is most basic—that is, has an electron deficiency such as would be produced by an electron attractive group in the benzene ring (Rodebush and Buswell, 1939).

This scheme can also provide an explanation of the greater structural specificity which the choline ether series seem to possess as compared with choline esters and the corresponding ketones; the carbonyl oxygen of esters and ketones would form a covalent bond which, once it had reacted, would fix this part of the molecule; on the other hand, the hydrogen bond reacting with the ether group is a much weaker bond, and would make the reaction of the substance with its locus on the active surface of the ganglion more dependent on the close fitting of the rest of the molecule.

Summary

1. Pharmacological actions of a series of derivatives of benzoylcholine have been studied and their potencies compared with the potency of the parent compound.

2. The actions of benzoylcholine and *p*-methoxybenzoylcholine have been shown qualitatively to resemble the action of nicotine and the " nicotine-like " action of acetylcholine on the blood pressure of the cat.

3. The activity of this series shows a correlation with the substituent constants for members of the series: this is taken as evidence for the existence of carbonyl receptor groups on the active surface of the ganglion.

4. These results are compared with those obtained in a study of a series of derivatives of choline phenyl ether.

5. A type of receptor is proposed that might explain these results.

I should like to express my thanks to Dr. H. C. Stewart for his interest and encouragement, Dr. P. A. Nasmyth and Professor W. D. M. Paton for the help that they have given me during the course of this work, and Dr. P. Armitage for his advice and for the statistical analysis.

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THE MODE OF ACTION OF SOME NON-SPECIFIC ACETYLCHOLINE ANTAGONISTS

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Antihistamines such as 2(*N*-benzyl-*N*-phenyl-aminomethyl)-iminazoline hydrochloride (antazoline hydrochloride) and *N*-dimethyl-aminoethyl benzhydryl ether hydrochloride (diphenhydramine hydrochloride), have been shown to possess many side-effects (Dutta, 1949), including local anaesthetic effects, antagonism of the depressant action of acetylcholine on the heart, depression of the maximal rate at which the isolated rabbit auricles will respond to electrical stimulation, antagonism of acetylcholine on isolated frog rectus abdominis muscle, and depression of the contraction of the nictitating membrane in response to preganglionic stimulation. Often, the concentrations of antihistamines required to produce such effects considerably exceed those necessary to produce the more specific effects against histamine, so that their modes of action are obscure. Marshall (1955a) claimed to have shown that the antagonism between many antihistamines and histamine on the guinea-pig ileum is competitive, although the criteria upon which competition was judged are not in entire agreement with those adopted in this paper.

A report is presented upon a preliminary investigation into the mode of action of two series of antihistamines as antagonists of acetylcholine (ACh) on the isolated guinea-pig ileum.

METHODS

The structure of the two series of compounds used in this work appear in Tables I and II.

The lever used throughout this work was constructed according to the dimensions suggested by Schild (1947), and was not allowed to move more than 30° from the horizontal. These precautions ensure that all measurements of the heights of excursions from the kymograph paper are linearly related to the actual shortening of the muscle.

Measurement of Anti-Acetylcholine Activity.—In order to determine the anti-ACh activity of these compounds, pA_2 and pA_{10} values (Schild, 1947) were measured against ACh on the isolated guinea-pig ileum. The tissue was suspended in an oxygenated Tyrode solution which was contained in the 2.0 ml. bath of an automatic assay apparatus of the type described by Boura, Mongar, and Schild (1954). Doses of ACh bromide (usually 0.0125 μg. base/ml. as basic dose) were added every 2 min. and antagonists were allowed to remain in contact for 10 min., represented by 5 complete cycles of the apparatus. pA_2 and pA_{10} values for each compound were not always determined on the same sample of ileum. Samples of ileum which did not recover fully from the effects of the antagonists were discarded, and fresh samples were used for the next test (Schild, 1947). Four determinations of pA_2 and four of pA_{10} were carried out for each substance using samples of intestine from different guinea-pigs.

Concentration-action Curves.—In order to determine the validity of the *p*A method as applied to the active-drug/antagonist systems studied, concentration-action curves were plotted for the active substance, ACh or histamine, first alone and then after equilibration to a fixed concentration of antagonist (Schild, 1949). The importance of such curves is discussed later in this paper, and the experimental procedure adopted to obtain them was as follows.

Fresh guinea-pig ileum samples were set up in the automatic isolated organ bath in the normal manner, and were stimulated every two minutes by a constant submaximal dose of acetylcholine bromide, until three consecutive contractions of approximately equal height were obtained. Usually between ten and twenty stimulations were required before this state was reached. Either 0.25 μg. or 0.05 μg. ACh/ml. was used, choice depending upon the sensitivity of the preparation. When constant responses had been obtained, six graded concentrations of ACh were administered, set up in the automatic bath in two groups of three concentrations. Within each group, the doses were administered in random order; three responses to each dose were obtained.

The concentrations of ACh usually employed and their normal groupings were, in μg./ml.:

(a) 0.125, 0.05, 0.10.

(b) 0.025, 0.075, 0.15.

At the end of each set, a single contraction was elicited by the addition of 1.0 μg. ACh/ml. This concentration of ACh always produced a maximal contraction of the muscle in the absence of antagonist, although sometimes

this was achieved with only 0.5 μg./ml. Maximal or near maximal doses had a detrimental effect upon the muscle, causing diminished responses to subsequently administered sub-maximal doses. Care was taken to eliminate any errors resulting from this, by ascertaining that the responses to lower concentrations of spasmogen were unaffected after maximal or near maximal doses. When this was not so, the muscle was allowed to recover before proceeding with the experiment.

Data for the curves in the presence of antagonist were obtained in the following manner. The wash fluid was changed from normal Tyrode to Tyrode containing the antagonist in the test concentration, which was usually in the region of the pA_{10} level. The gut was stimulated by a higher dose of ACh, usually 0.5 μg./ml., containing the test concentration of antagonist, until equilibrium between the antagonist and the receptors was obtained. This was indicated by a levelling out of the heights of contraction, which, before equilibrium, had tended to decrease with time of contact between antagonist and muscle. Equilibrium was usually reached within 15 min.

At this stage, the preparation was stimulated by six doses of ACh bromide, added in two groups of three doses as before, each individual dose being administered three times. The doses of ACh were adjusted according to the amount of depression produced by the antagonist, but they were all correspondingly higher than those used to plot the curve for active drug alone, thus causing a shift of the second curve along the log dose abscissa. The maximal height of contraction in the presence of antagonist was determined by stepping up the dose of ACh until a further increase produced no difference in the height of contraction. It was not always practicable to administer each of these higher doses three times, due to the paralysing effect of repeated higher doses of ACh upon the muscle. As before, the muscle was allowed to recover from the effect of the higher doses before further tests were carried out. All stimulant solutions used to obtain data for the second curve contained the test concentration of antagonist.

The heights of contraction were measured in millimetres, and the mean height at each dose level was computed and expressed as a percentage of the maximal height in the absence of the antagonist. For convenience in plotting, the doses of stimulant were multiplied by one hundred before conversion to logarithmic form. They were plotted as abscissae against the corresponding percentage effects as ordinates.

Test for Parallelism.—To apply a statistical test for parallelism to the concentration-action curves, it was necessary to convert the curves to linear form.

To explain the parallelism frequently observed with many antagonist systems, Gaddum (1937) derived the equation:

$$K_1C_1=(1+K_2C_2^n)\cdot\frac{y}{100-y}$$

where C_1 is the concentration of active drug, C_2 the concentration of antagonist, and y=the effect of the active drug.

From this equation:

$$C_1=\frac{1+K_2C_2^n}{K_1}\cdot\frac{y}{100-y}$$

whence

$$\log_{10}C_1=\left[\log_{10}\frac{1+K_2C_2^n}{K_1}\right]+\left[\log_{10}\frac{y}{100-y}\right]$$

A plot of $\log_{10}C_1$ against $\log_{10}\frac{y}{100-y}$ should therefore be linear. This appeared to be so for all the concentration-action curves obtained. The significance levels of the calculated linear regressions were found to be less than 0.20 for all curves (see Table IV).

The significance of the absence of parallelism was calculated by an analysis of variance.

Composition of Tyrode Solution.—NaCl, 8.0 g.; KCl, 0.2 g.; $CaCl_2$, 0.2 g.; $MgCl_2$, 0.01 g.; NaH_2PO_4, 0.05 g.; $NaHCO_3$, 1.0 g.; dextrose, 1.0 g.; distilled water to 1 litre.

Treatment of Glassware.—Discrepancies in the experimental results due to adsorption of the basic antihistamines on to the glassware (Marshall, 1955a) were avoided by thorough washing in hot tap water followed by immersion in 33% v/v commercial nitric acid overnight; further treatment with hot tap water and distilled water preceded subsequent use. The glassware of the automatic assay apparatus was treated in the same manner.

Results

Tables I and II contain the results of pA_2 and pA_{10} determinations on two series of compounds which are relatively specific antihistamines. The probability (P) was calculated that the differences between (pA_2–pA_{10}) values for two compounds A and B could have occurred by chance. Values of t and P for the halogenated members of two series and their respective non-halogenated "parent" compounds are shown in Table III; values of t were calculated from the formula

$$t=\pm\frac{(pA_2-pA_{10})_A-(pA_2-pA_{10})_B}{\sqrt{(eA_2)^2+(eA_{10})^2+(eB_2)^2+(eB_{10})^2}}$$

in which eA_2 is the standard error of the mean pA_2 for compound A, and eA_{10} is the standard error of the mean pA_{10} for this compound; eB_2 and eB_{10} are the corresponding values for the second compound, B.

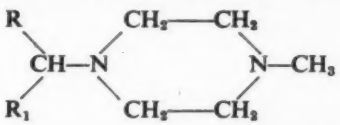
Piperazine Derivatives.—This series provides an interesting illustration of the extent to which relatively small variations of chemical structure can affect pharmacological activity. Table I shows the composition and pA_2 values for the members of this series. Compounds A2285, A2342, chlorcyclizine and A2824 differ from the parent compound only in the possession of a halogen atom.

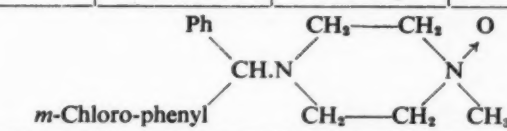
TABLE I

STRUCTURES AND ACTIVITIES OF PIPERAZINE DERIVATIVES

Antagonism to acetylcholine expressed as pA_2 values (standard deviations in parentheses; all values are means of four determinations)

General formula:



Serial No.	R	R_1	Salt	pA_2	pA_{10}	pA_2–pA_{10}
A1198 ..	Phenyl	Phenyl	HCl	6·40 (0·03)	5·79 (0·20)	0·61
A2285 ..	*p*-Bromo-phenyl	,,	,,	5·89 (0·09)	5·54 (0·19)	0·35
A2342 ..	*p*-Fluoro-phenyl	,,	,,	6·08 (0·05)	5·62 (0·12)	0·46
Chlorcyclizine	*p*-Chloro-phenyl	,,	,,	5·86 (0·08)	5·25 (0·17)	0·61
A2824 ..	*m*-Chloro-phenyl	,,	,,	6·46 (0·12)	5·72 (0·11)	0·74
A2917 ..	*p*- ,, ,,	2-Thiophen	$2(COOH)_2$	5·86 (0·09)	5·36 (0·16)	0·50
A4826 ..			$2(COOH)_2$	Anti-acetylcholine activity negligible		
A2688 ..	*p*-Methyl-phenyl	Phenyl	HCl	5·75 (0·09)	5·40 (0·11)	0·35

In the first three halogenated compounds, the halogen is in the para position, in the fourth in the meta position of one phenyl ring. The pA_2 values of all three para-halogenated compounds are distinctly lower than that of the parent compound, and only the fourth (meta-halogenated) compound has pA_2 of the same order as that for the parent substance.

Para-methylation of one phenyl ring (compound A2688) appears also to lower the pA_2 value compared with the parent compound, which suggests that this effect is not confined to halogenation. Statistical analysis of the results has shown that the differences in pA_2 and pA_{10} values of halogenated and non-halogenated compounds are not always significant. The pA_{10} values for A2285 and A2342 are not significantly different from that of A1198. With these exceptions, however, halogenation does appear to alter anti-ACh activity in this series, to an extent considered to warrant further investigation.

(pA_2–pA_{10}) *Values.*—(pA_2–pA_{10}) values for these antihistamines are interesting in that they are all below 0.95, the theoretically-derived value for first order competitive antagonists (Schild, 1947). With the exception of chlorcyclizine, the para halogenated compounds showed smaller (pA_2–pA_{10}) differences than the parent compound, though statistically such differences were not always significant (Table III). The compound with the halogen in the meta position (A2824), on the other hand, shows a (pA_2–pA_{10}) difference of the same order as that for the parent substance. The very low (pA_2–pA_{10}) values shown by several of these compounds are considered to indicate a direct depressant effect upon the muscle. There is some evidence for this view. Papaverine, a substance known to depress the muscle directly, was found to have pA_2 of 5.12 and pA_{10} of 5.17 giving the extremely small value for (pA_2–pA_{10}) of 0.05.

These results indicated that there might be an essential difference in the mode of action of the halogenated compounds compared with that of the parent substance.

A further interesting result is shown in Table I. Compound A4826 differs from the parent compound A1198 in two respects. Firstly, A4826 possesses a meta-chlorine atom in one phenyl ring of the benzhydryl moiety, and secondly, the nitrogen atom in the piperazine structure of this compound is linked co-ordinately to an oxygen atom, whereas, in the parent compound, the electron pair of the corresponding nitrogen atom remains free. A4826 had negligible anti-acetylcholine activity. A concentration corresponding to two hundred times that of the pA_2 for the parent compound had only slight activity against the double dose of ACh. (0.025 μg./ml.) The inference is, then, that the tertiary nitrogen atom of the piperazine structure in these compounds is essential to anti-acetylcholine activity. Fixation of the free electron pair from this atom in the formation of a co-ordinate bond (as in A4826), is

TABLE II

STRUCTURES AND ACTIVITIES OF *N*-DIMETHYL-*N*(*n*-PROPYL) AMINE DERIVATIVES

Antagonism to acetylcholine expressed as pA_2 values (standard deviations in parentheses; all values are means of four determinations)

Compound	R_1	R_2	pA_2	pA_{10}	pA_2–pA_{10}
Prophenpyridamine ("Trimeton" Schering Corp.)	Phenyl	2-Pyridyl	5·54 (0·03)	4·61 (0·15)	0·93
Chlorprophenpyridamine ("Chlortrimeton," Schering Corp.) ..	*p*-Chloro-phenyl	,,	5·59 (0·13)	4·99 (0·08)	0·60

accompanied by considerable decrease of anti-acetylcholine activity.

Prophenpyridamine and Chlorprophenpyridamine.—Although the pA_2 values of these compounds were approximately of the same order, the pA_{10} values and consequently the (pA_2–pA_{10}) differences were found to differ significantly (Tables II and III). The (pA_2–pA_{10}) difference of the parent compound was 0.93, the value for a first order competitive antagonist, whereas the value for chlorprophenpyridamine was only 0.60, again an indication that the halogenated member acts in a different manner from the non-halogenated compound.

TABLE III

STATISTICAL TREATMENT OF RESULTS TO TEST EFFECT OF HALOGENATION ON (pA_2–pA_{10})

	Difference Between (pA_2–pA_{10}) for A1198 and Designated Compound	Degrees of Freedom *n*	*t*	*P*
A2285 ..	0·26	12	1·5	$0·1<P<0·2$
A2342 ..	0·15	12	1·25	$0·2<P<0·3$
Chlorcyclizine	0·00	12	—	—
A2824 ..	0·13	12	1·02	$0·3<P<0·4$

Difference Between (pA_2–pA_{10}) for Prophenpyridamine and Chlorprophenpyridamine	Degrees of Freedom *n*	*t*	*P*
0·33	12	3·27	<0·01

The results for these two groups of antihistamines indicated that there might be an essential difference in the mode of action of the halogenated compounds compared with that of the parent compound, and suggested the necessity for further investigation.

Concentration-action Curves

Gaddum's formula (1937) for drug antagonism is based on the experimental fact that concentration-action curves are frequently parallel when plotted first in the absence and then in the presence of a constant concentration of antagonist. This fact is also the basis of the *p*A measure, for parallelism on a log dose abscissa indicates that *p*A is independent of the height of contraction, and therefore of the initial concentration of active drug (Schild, 1949). In addition, concentration-action curves may be used as a test of competitiveness in active-drug-antagonist systems (Van Maanen, 1950); if the curves in the presence and absence of the antagonist are parallel, and if the same maximal height of contraction can be obtained in either case, the antagonist is likely to be acting by a competitive mechanism.

It was considered necessary, therefore, to plot concentration-action curves for acetylcholine on the isolated guinea-pig ileum, with the halogenated and non-halogenated "parent" compounds of the two series used in this investigation (Figs. 2–8). By this means, evidence could be obtained concerning the extent to which the *p*A method is applicable to antihistamine/acetylcholine systems, and also the mode of action of these substances when antagonizing ACh.

A preliminary experiment was carried out with atropine and ACh in order to determine what type of concentration-action curve this highly specific antagonist would produce.

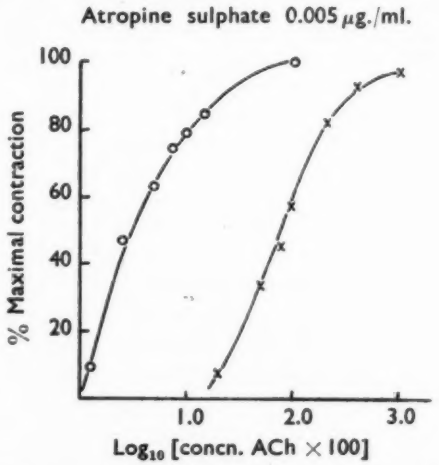


FIG. 1.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine in the presence of atropine sulphate 0.005 μg./ml.

Fig. 1 shows the curves obtained. The second was plotted after equilibration to 0.005 μg. atropine/ml. When analysed statistically the curves showed no significant deviation from parallelism (Table IV) and the effect of atropine was reversible. It is concluded, therefore, that the antagonism is competitive.

TABLE IV

STATISTICAL TEST FOR PARALLELISM ON CONCENTRATION-ACTION CURVES

Antagonist	Significance of Regression for ACh Alone	Significance of Regression for ACh Plus Antagonist	Significance of Absence of Parallelism	Parallel + Non-Parallel
Atropine ..	0 05	<0·001	>0·20	+
Prophenpyridamine ..	<0·01	<0·01	>0·20	+
Chlorprophenpyridamine..	<0·001	<0·05	<0·001	−
A1198 ..	<0·20	<0·001	>0·20	+
A2285 ..	<0·01	<0·50	<0 01	−
A2342 ..	0 001	<0·20	<0 05	−
A2824 ..	<0·01	<0·20	<0·20	−
Chlorcyclizine	<0 01	<0·20	<0·05	−

Antihistamines

It was found that the concentration-action curves for A1198 and prophenpyridamine, the non-halogenated parent compounds, were parallel, and that the effect of the antagonists could be reversed by suitably increasing the ACh concentration (Figs. 2 and 7). The conclusions which may be drawn are, firstly, that these compounds are competitive antagonists of ACh on this preparation, and, secondly, that *p*A is independent of the height of contraction.

The halogenated antihistamines (Figs. 3, 4, 5, 6, 8), on the other hand, produced an effect upon the muscle which could not be reversed by ACh. It may therefore be concluded that their action is non-competitive on this preparation. Furthermore, the pA_x values recorded for these compounds, and particularly the pA_{10} values, were subject to errors arising from the dependence of *p*A upon the height of contraction.

The curves for the halogenated compounds were not of the type required by the mass law equation derived by Schild (1954) for non-competitive antagonism, which requires that the slopes and maxima of the curves should fall off with increasing concentrations of antagonist.

It was considered that the depression of the maximal response shown with these compounds

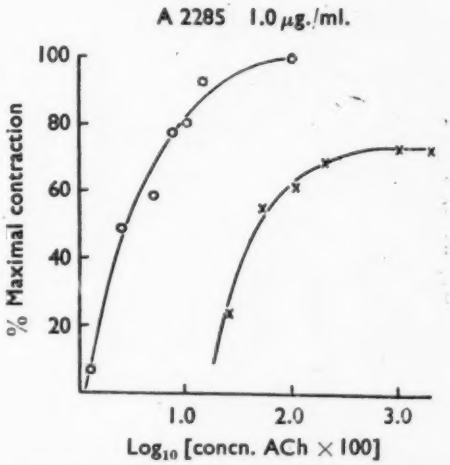


FIG. 3.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to A2285, 1.0 μg./ml.

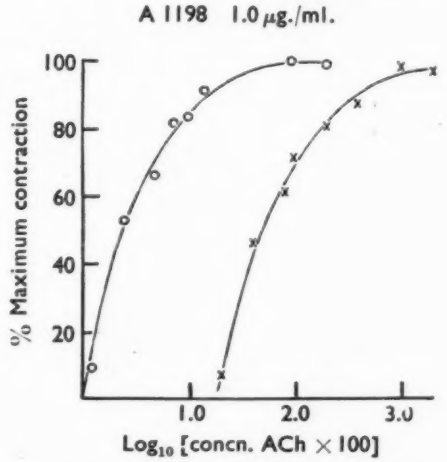


FIG. 2.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to A1198 1.0 μg./ml.

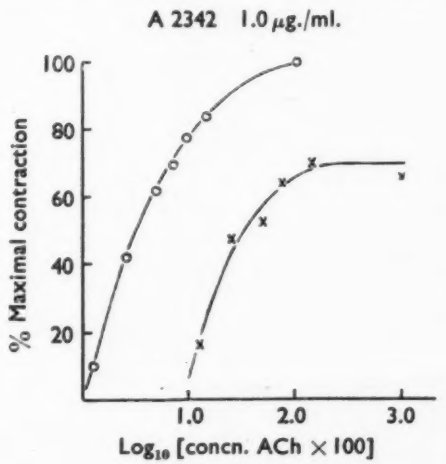


FIG. 4.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to A2342, 1.0 μg./ml.

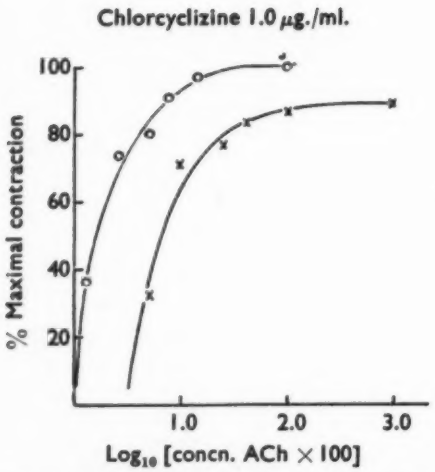


FIG. 5.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to chlorcyclizine, 1.0 μg./ml.

might be due to the high concentrations used, and that lower ones might not show this effect. An investigation of the effects of lower concentrations of antagonists was therefore necessary. It seemed reasonable to suppose that a direct depression of the muscle would be apparent whatever the spasmogen, and hence, in experiments in which the concentration of antihistamine was insufficient to affect responses to ACh, histamine was used as the spasmogen.

Fig. 9 shows the concentration-action curve for chlorcyclizine at a concentration of 10 ng./ml., and Fig. 10 that for 100 ng./ml., against histamine. Figs. 11 and 5 show the corresponding curves for concentrations of 100 ng./ml. and of 1.0 μg./ml.

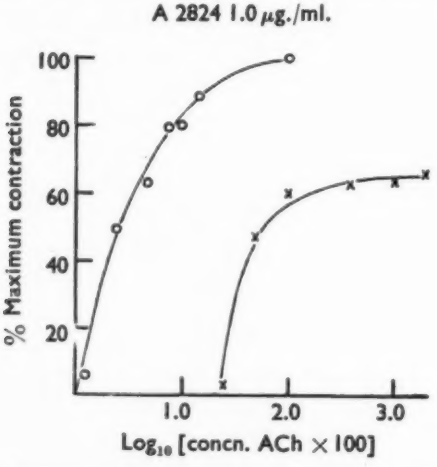


FIG. 6.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to A2824, 1.0 μg./ml.

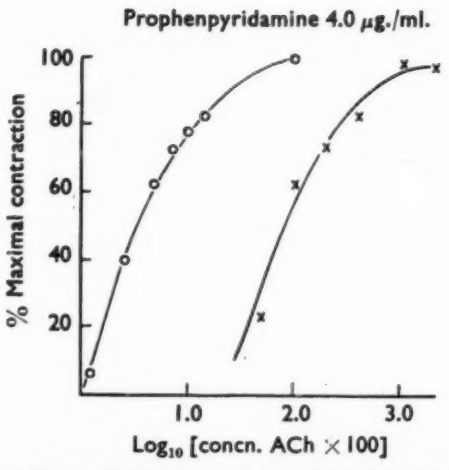


FIG. 7.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to prophenpyridamine, 4.0 μg./ml.

against ACh. The curves show that at concentrations of 10 ng./ml. and 100 ng./ml. there is no direct depression of the muscle, and that a further increase of the chlorcyclizine concentration to 1.0 μg./ml. produces this effect.

DISCUSSION

Non-specific Antagonism

The results presented show that antagonisms of a non-specific nature, such as those between ACh and antihistamines, may be competitive or non-competitive. This depends firstly upon the structure of the antagonist, and secondly upon the relationship between the concentration of antagonist required to produce a reasonable degree of antagonism, and the minimum concentration at which side effects,

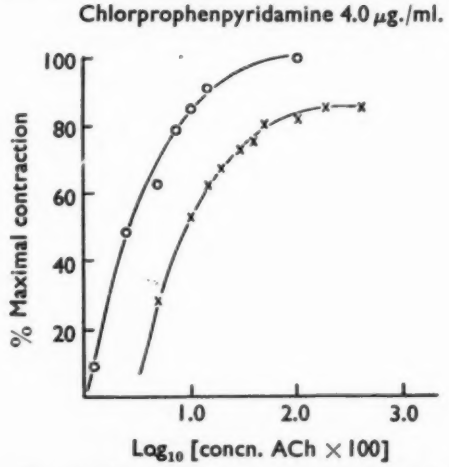


FIG. 8.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to chlorprophenpyridamine, 4.0 μg./ml.

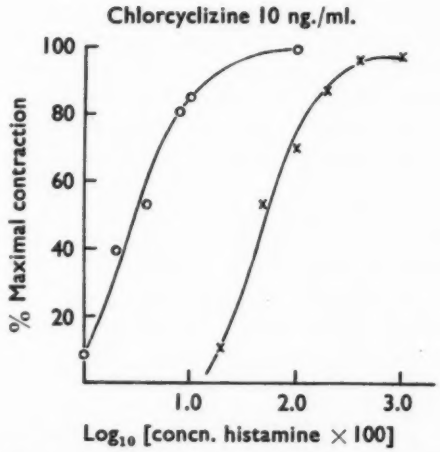


FIG. 9.—Concentration-action curves for histamine on the isolated ileum of the guinea-pig. O——O curve for histamine alone. ×——× curve for histamine after equilibration to chlorcyclizine, 10 ng./ml.

such as non-specific depression of the muscle, occur.

Marshall (1955a) obtained (pA_2–pA_{10}) values greater than 0.95 for some antihistamines against histamine on the guinea-pig ileum. These were considered to be due to "wastage" upon non-specific receptors, such as those for ACh. With higher concentrations of antagonists, such as those required for pA_{10} measurements, it was supposed that so much antagonist was adsorbed upon ACh receptors, that the effective concentration of antagonist in the region of the histamine receptors was reduced. Consequently, a greater increase in the concentration of antagonist was required than that theoretically expected for "competitive" antagonists between pA_2 and pA_{10}. In an attempt to confirm this view, Marshall (1955a) determined

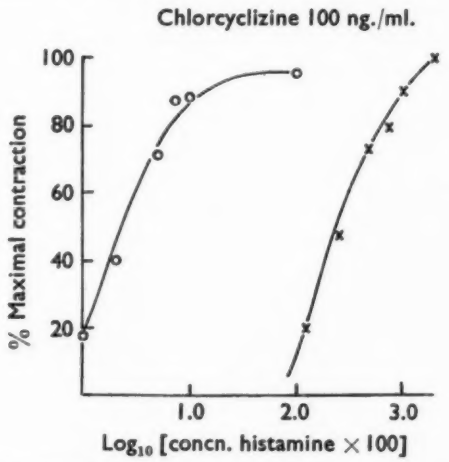


FIG. 10.—Concentration-action curves for histamine on the isolated terminal ileum of the guinea-pig. O——O curve for histamine alone. ×——× curve for histamine after equilibration to chlorcyclizine, 100 ng./ml.

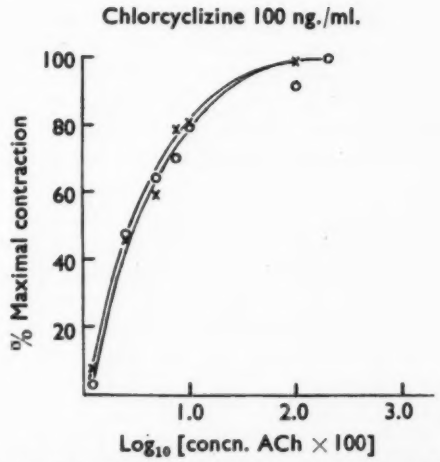


FIG. 11.—Concentration-action curves for acetylcholine on the isolated terminal ileum of the guinea-pig. O——O curve for acetylcholine alone. ×——× curve for acetylcholine after equilibration to chlorcyclizine, 100 ng./ml.

(pA_2–pA_{10}) against histamine for some of the antihistamines which had shown "abnormally high" values, using atropine sulphate in the bathing fluid in order to block the ACh receptors and prevent the access of the antihistamines to these receptors. Only with chlorcyclizine was the expected effect of lowering (pA_2–pA_{10}) to a value nearer 0.95 achieved. In all cases, the actual values of pA_2 and pA_{10} were lowered, some by as much as 0.46 pA units. This observation seems to suggest that, when atropine blocks the ACh receptors, the antihistamines are less effective antagonists of histamine. This is contrary to the concept that fewer antihistamine molecules are then being "wasted" upon ACh receptors.

In this connexion, the experiments described in this paper with a range of concentrations of chlorcyclizine against histamine and acetylcholine are interesting (Figs. 9–11). With 100 ng. chlorcyclizine/ml. (Fig. 10) a hundred-fold increase of histamine was necessary to reproduce a response equal to 50% of the maximum. Even at this high concentration of chlorcyclizine, there was no demonstrable antagonism to ACh (Fig. 11). From this, it may be inferred that there was no significant "wastage" of chlorcyclizine molecules on to ACh receptors.

Marshall's hypothesis is open to criticism on theoretical grounds also. If one assumes a pseudo-monomolecular reaction between the receptors and active-drug molecules on the one hand and antagonist molecules on the other, one must also assume that the effective concentration of receptors is relatively small (Van Maanen, 1950). Hence, the combination of either drug with the receptors would not alter the effective concentration of these substances in the extra-receptor fluid. Accepting this,

it is difficult to see how adsorption of antihistamine molecules upon ACh receptors can affect their concentration and adsorption at the histamine receptors, especially since it has been shown that with chlorcyclizine the amount " wasted " on to ACh receptors is insufficient to affect the responses to ACh.

Values of (pA_2–pA_{10}) significantly less than 0.95 were considered by Marshall (1955b) to indicate non-competitive antagonism. However, it has been shown in this paper that antagonists with (pA_2–pA_{10}) other than 0.95, such as A1198 and atropine, may still give parallel curves. Marshall obtained a (pA_2–pA_{10}) value of 0.73 for atropine sulphate against acetylcholine on the isolated guinea-pig ileum, and concluded that the antagonism was non-competitive. The concentration action curves for acetylcholine with and without atropine (Fig. 1) are parallel and there is no depression of the maximum, suggesting that the antagonism is competitive. The same applies to A1198 and ACh (Table I and Fig. 2). Hence, it cannot be concluded that antagonists for which (pA_2–pA_{10}) is significantly less than 0.95 are non-competitive. In fact, with antagonist/agonist systems to which the *p*A method has been shown to be applicable by concentration-action curves, (pA_2–pA_{10}) gives only an indication of " n " in the Gaddum (1937) equation, " n " being the number of molecules of antagonist required effectively to block one receptor. Where very low (pA_2–pA_{10}) values are obtained, as with papaverine in the present work, it seems unlikely on logical grounds that a competitive antagonism exists.

It would seem reasonable to expect that one molecule of atropine is capable of blocking one ACh receptor to the exclusion of one molecule of ACh. The failure of atropine to produce values consistent with this hypothesis may either be due to the fact that we are not in fact dealing with a first order reaction, or, as is at least equally probable, the discrepancy may be due to the failure of the Gaddum (1937) equation to express the antagonism exactly. In the absence of any *a priori* reason to suppose that active and antagonistic drugs are reacting chemically together, or that the antagonistic drug alone is capable of exerting a physiological effect which is directly opposed to that of the active drug, the concentration-action curve method used in this work has some value in helping to decide whether an antagonism is competitive or not.

Effects of Halogenation

The present work has revealed the interesting fact that relatively small alterations in structure, such as para-halogenation, have the ability to alter not only the potency of an antagonistic drug but also its mode of action. This has been shown for two series of antihistamines, when tested for their ability to antagonize the ACh-induced spasm of the isolated guinea-pig ileum. With the piperazine antihistamines, para-halogenation, as in compounds A2285, A2342, and chlorcyclizine, appeared to alter the potency of the antagonists more than meta-halogenation, as in A2824, but both substitutions altered the mode of action of the antagonists as shown by the concentration-action curves. The concentration-action curves for halogenated antihistamines of both series provide two pieces of information. In the first place the *p*A measurements are not reliable, particularly the pA_{10} values, where higher concentrations of antagonists are necessary. Secondly, in distinction to the non-halogenated " parent " compounds, prophenpyridamine and A1198, which are competitive antagonists of ACh, the halogenated members are non-competitive. The concentration-action curves for these compounds are different from those described by Zadina (1947) and Zadina and Kriz (1948) and also from those described by Guarino and Bovet (1949), and must therefore represent a different type of antagonism.

The depressed maximal response to ACh seen with the halogenated antihistamines studied in this work might be explained in three ways. Firstly, the halogenated compounds might act irreversibly upon some of the higher threshold ACh receptors. Their effect would then be the inactivation of some of these receptors. This explanation would still hold good even if only 50% of the receptors were occupied by ACh during a maximal contraction. The second possibility is that the halogenated antihistamines depress the muscle directly and not by a receptor mechanism. The manner in which halogenation produces this effect could possibly be that the toxicity of the compounds to the smooth muscle is increased by the introduction of an electronegative atom into the structure, which lowers the threshold concentration for direct depression of the muscle. Finally, there remains the possibility that the halogenated compounds, but not the non-halogenated compounds, are able to antagonize the ganglionic component of the contractions of the guinea-pig ileum in response to ACh (Feldberg, 1950). This aspect of their mode of action has yet to be investigated.

Effects of Methylation

It is possible that the effects ascribed to halogenation may be produced by other substitutions

as well. For example, the introduction of a methyl group into the para-position of one phenyl ring in the piperazine derivatives as in A2688, had the effect of lowering the (pA_2–pA_{10}) difference for the compound compared with the " parent " compound, a similar effect to that produced by para-halogenation. However, the analogy between the effects of these two substitutions can be taken no further in the absence of additional evidence upon the mode of action of the methylated compound.

A4826.—An interesting point in structure-activity relationships is brought out by the results obtained with this compound. The incorporation of the lone electron pair of one of the piperazine nitrogen atoms into a co-ordinate linkage with an oxygen atom (Table I) results in an almost complete loss of anti-ACh activity. This suggests that the free electron pairs of the piperazine nitrogen atoms must remain free in order that the compounds may exhibit anti-ACh activity.

Summary

1. The activity of two series of antihistamines against the ACh-induced spasm of the isolated guinea-pig ileum were investigated by the *p*A method. In both series of antihistamines, the introduction of a halogen atom in the para-position of one phenyl ring had a marked effect upon the activity of the compounds as ACh antagonists. Meta-halogenation had less effect. There is evidence that methylation may have similar effects.

2. The modes of action of these substances and the validity of the *p*A measurements were investigated by plotting concentration-action curves for ACh in the absence and then in the presence of the antagonists. It is concluded that the introduction of halogen atoms into these two types of compound produces non-competitive antagonists of ACh, their non-halogenated counterparts being competitive.

3. Atropine is probably a competitive antagonist of ACh on the isolated guinea-pig ileum.

4. Criteria by which the mode of action of antagonists may be judged are discussed.

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ASSESSMENT OF DEPRESSOR ACTIVITY AND MYDRIATIC ACTIVITY OF HEXAMETHONIUM ANALOGUES

BY

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Most of the techniques used in our laboratory for the pharmacological study of compounds synthesized as possible depressor agents are well known; their merits and demerits have received much attention. There are some, however, which have been used comparatively seldom for studies of this sort. Therefore, in the hope that our experience may be of use to other workers in the field, we are describing certain types of experiment which we perform frequently.

Since most of the compounds which we have tested resemble hexamethonium fairly closely in chemical structure, and since the latter is believed to lower blood pressure mainly—perhaps wholly—by blocking autonomic ganglia, we have been interested in techniques for assessing ganglion-blocking activity. The fact that changes in pupil size can be readily measured with mice by the Pulewka technique has been exploited by several workers (Wien and Mason, 1951; Edge, 1953; Schwarzacher and Stumpf, 1954), who have shown how the mydriatic effect of hexamethonium-like compounds may be used as a measure of their ganglion-blocking activity. Further details about the measurement and comparison of the mydriatic effects of hexamethonium-like compounds are given below.

Although a technique depending essentially upon the measurement of the degree of ganglion-blockade obtained at a particular site may furnish valuable information about the potency of hexamethonium analogues, the possibility that at least some of the latter may lower blood pressure by mechanisms other than ganglion-blockade makes it imperative that their depressor activity should be assessed mainly by a direct method. We have therefore tried to develop a reliable technique for comparing depressor effects in animals under conditions not far removed from those obtaining clinically. The "ear capsule" technique introduced by Grant and Rothschild (1934) for measuring the blood pressure of the unanaesthetized rabbit appealed to us for the following reasons: (1) No complications due to the anaesthetic arise; (2) because measurements are made with the animal upright, a ganglion-blocking agent produces a much greater depressor effect than it would were the animal lying on a table (owing to abolition of the reflexes which oppose gravitational pooling of the blood); (3) it is possible, in order to avoid changes in sensitivity due to cumulation, or to the development of tolerance, to inject compounds at relatively infrequent intervals (this is not readily accomplished in acute non-survival experiments); (4) it is easy to inject drugs intravenously into rabbits, which are comparatively cheap and readily available.

METHODS

Rabbits.—White rabbits of an inbred strain and weighing 1.5–2.0 kg. were used. Their blood pressures were recorded with a modified Grant ear capsule. This capsule differed from that described by Grant and Rothschild (1934) mainly in that the membrane—for which sausage skin is suitable—was transilluminated from a torch bulb mounted within the pressure chamber. The rabbits were placed in an electrically heated box, which was warmed to, and then maintained at, body temperature before any measurements were made. The minimum pressure required to occlude the central artery of the right ear was recorded once or twice a minute until steady readings were obtained. The drug (in 0.3 ml. isotonic solution) was then injected into the marginal vein of the left ear. Injections of potent drugs were made no more often than once every 3 days. Each rabbit was given alternately a standard drug (hexamethonium or pentolinium) and the compound under investigation, and pairs of rabbits were used to improve the comparison of the two compounds by a "cross-over" test.

Mice.—Mydriatic effects were measured by Pulewka's method (Burn, 1950). The mice were kept for at least 30 min. in separate beakers under bright illumination before their pupil diameters were measured with a dissecting microscope containing an arbitrary scale in the eyepiece. To make the illumination as uniform as possible, the beakers containing the mice were placed beneath long low-power fluorescent tubes and on top of glossy white paper. Drug solutions were given intraperitoneally, and

changes in pupil size were measured at fixed times afterwards.

Preliminary experiments were performed with batches of 20 mice divided into groups of 5. One mouse of each group was given a control injection of 0.9% NaCl (0.01 ml./g. body weight); the others were given 20 mg./kg. (2 mice) or 40 mg./kg. (2 mice) of one of the compounds under investigation dissolved in saline. One group of each batch received the same dose of hexamethonium bromide. Later, saline controls were dispensed with, and the compounds were given in distilled water.

In further experiments with the more active compounds, 10 of a group of 20 mice were given hexamethonium bromide at two different dose levels and 10 the compound under investigation, also at two different dose levels. The estimate of equipotent doses thus obtained was used as the basis of a second similar experiment designed to give a better comparison.

Compounds.—Most of the compounds referred to by code name in Table II are of structure N–X–N, 2Br, where X is a hydrocarbon chain usually containing 5–7 carbon atoms joining the two substituted ammonium groups. Structural details of such compounds are given in Table I. "Ecolid" (Ciba Su-3088) is ethylene-1-trimethylammonium-2-(*N*-methyl-4′5′6′7′-tetrachloro)-*iso*indolinium dichloride. The

TABLE I
STRUCTURAL DETAILS OF COMPOUNDS

Code Name	Hydrocarbon Residue	Cationic Groups
Pentolinium	Pentamethylene	*N*-Methyl-pyrrolidinium
"Gaplegin"	Hexamethylene	Ethyl-dimethylammonium
NP328 ..	,,	*N*-Methyl-morpholinium
NP73 ..	Pentamethylene	2-Hydroxyethyl-dimethylammonium
Hexamethonium	Hexamethylene	Trimethylammonium
NP325	,,	2-Hydroxyethyl-dimethylammonium
"Pendiomide"	$-CH_2CH_2N(CH_3)CH_2CH_2-$	Ethyl-dimethylammonium
NP266 ..	Pentamethylene	*N*-Methyl-morpholinium
NP263	,,	*N*-Ethyl-piperidinium
NP336 ..	Hexamethylene	*N,N*-Diethyl-anilinium
NP15 ..	Ethylene	2-Hydroxyethyl-dimethylammonium
NP270 ..	Pentamethylene	*n*-Propyl-dimethylammonium
NP277 ..	Tetramethylene	2-Hydroxyethyl-diethylammonium
NP251 ..	*p*-Xylylene	Trimethylammonium
NP253 ..	,,	2-Hydroxyethyl-dimethylammonium
NP255 ..	,,	*N*-Methyl-morpholinium

Burroughs Wellcome compound 288C53 has the structure

$$(C_6H_5)_2C{=}CH{-}(CH_2)_4{-}N(CH_3)_2{-}CH_2CH_2{-}N(CH_3)_2CH_2CH_3,\ 2I$$

Compound SC-1919, from G. D. Searle & Co., is *n*-

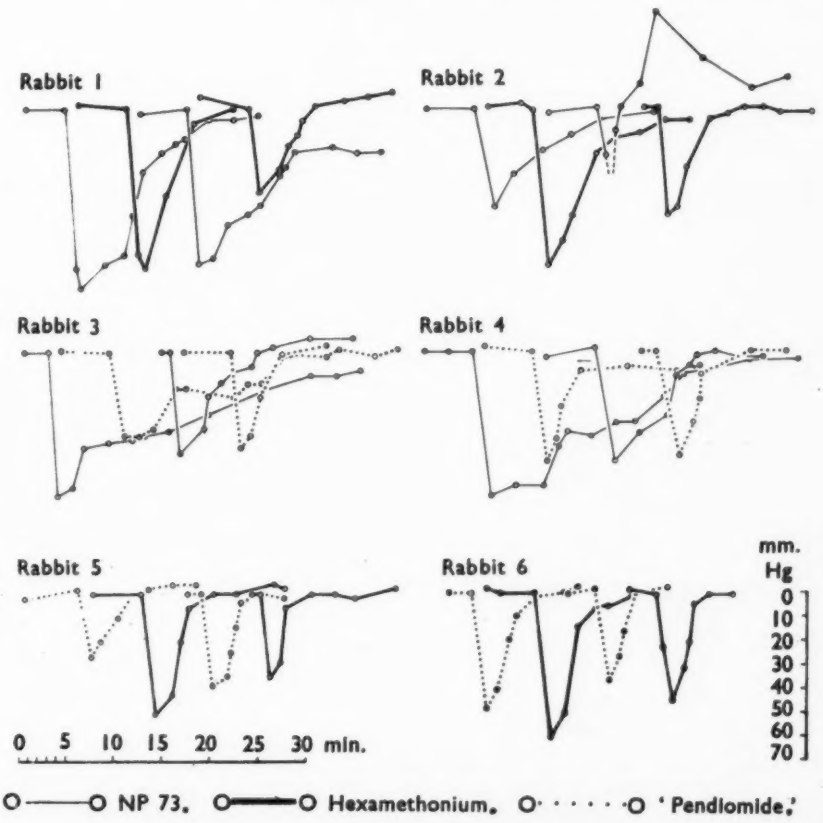

FIG. 1.—Effects on the blood pressure of unanaesthetized rabbits of quaternary ammonium compounds each given intravenously in a dose of 0.3 ml. of a 0.085M solution. The 4 injections given to each of the 6 rabbits were made at intervals of 3 days. The results of these cross-over experiments indicate that hexamethonium and NP73 (hexamethylene bis-2-hydroxyethyldimethylammonium-bromide) are almost equally active in lowering the blood pressure and distinctly more active than "Pendiomide" on a molar basis.

butyl-triethylammonium bromide. Compounds of the "NP" series were synthesized in the department.

Results

Experiments on Rabbits

After a few days' experience of the procedure most rabbits gave blood-pressure readings which did not vary by more than 5–10 mm. Hg from day to day, or when measured repeatedly over 30–60 min. A few rabbits which continued to give much more variable readings were rejected.

The reliability of each observer's results was checked by testing him with "unknown" solutions, some of which were inactive. No serious difficulty was experienced in detecting the active compounds, even when the observer was relatively unpractised in the technique, so long as the rabbits had been accustomed to the procedure.

A typical experiment with 6 rabbits gave the results illustrated in Fig. 1. Batches of 6 rabbits were generally employed, each rabbit being given 2 different compounds twice in 10 days. A semi-quantitative comparison of the depressor activities of a number of compounds was made by (1) judging from pilot experiments what doses are approximately as potent as the standard dose of hexamethonium, and (2) comparing the effects of these doses by a cross-over experiment of the type illustrated in Fig. 1. Typical results are given in Table II.

Table II

DEPRESSOR AND MYDRIATIC ACTIVITIES OF VARIOUS QUATERNARY AMMONIUM COMPOUNDS

Equidepressor doses in the rabbit were estimated as shown in Fig. 1. Equidepressor doses in man were calculated from clinical data (Smirk, 1952, and unpublished observations). Compounds for which no rabbit dose is given had negligible depressor activity when 25 μM were given intravenously

Code Name	Equidepressor Doses (μM)		Depressor Activity[1]	Mydriatic Activity[2]
	Rabbit	Man		
"Ecolid" ..	2·5	4	+++++	+++++
Pentolinium ..	5	6	++++	++++
288C53	10	40	+++	+++++
"Gaplegin" ..	20	25	++	++
NP328	23	—	++	+++
NP73	23	50	++	+++
Hexamethonium	25		++	++
NP325	30	—	++	++
"Pendiomide" ..	30	50	++	++
NP266	30		++	+++
SC–1919(=NP179)	50		+	++
NP263	50		+	+++
NP336			−	++
NP15			−	++(+)
NP270			−	++(−)
NP277			−	+
NP251			−	−
NP253			−	−
NP255			−	−
Choline[3]			−	−
Betaine[3]			−	−

[1] Based upon experiments on rabbits.

[2] Based upon preliminary experiments on mice. When subsequent experiments with larger numbers of mice altered the previous estimate of activity, the new estimate is given in parentheses.

[3] Used as controls in experiments with "unknown" solutions.

Although some experiments extended over several weeks no significant tolerance developed. But if hexamethonium was injected frequently (0.3 ml. of a 0.085M solution given subcutaneously twice a day), rabbits did develop some tolerance to it; within a fortnight the test dose of hexamethonium given intravenously would produce little or no fall of blood pressure.

Experiments on Mice

Variation in Control Readings.—Spontaneous variation in pupil size was small over one period of observations. The pupil diameters of the mice which received injections of saline alone remained practically constant. No change was observed with 140 out of 180 mice. Of the remaining 40, 37 showed only a slight (less than 20%) increase in pupil size and 3 an equally slight decrease. In view of these results, saline controls were dispensed with in later experiments.

Variation from mouse to mouse was not much greater. For more than 80% of the mice the initial pupil diameter was 3–5 (arbitrary) units, and all readings were within the range 2–7. Even when an ordinary bench lamp was used to keep the mice under bright illumination and the lighting was far from even, the variation from mouse to mouse was not obviously more than when the illumination was provided by long fluorescent tubes; but it was important to have as uniform illumination of the pupil as possible at the time of measurement. Less variation was obtained when an auxiliary light was attached to the microscope. To ensure a minimum initial value of the pupil diameter it was important to accustom mice to the bright illumination for 30–45 min. before taking the initial reading.

Mydriatic Effect of Hexamethonium.—Doses of the order of 10 mg./kg. of hexamethonium bromide produce considerable mydriasis in mice (Wien and Mason, 1951; Edge, 1953). Our mice were approximately as sensitive to hexamethonium as the mice used by these workers. We noted a 2–3-fold increase in pupil size with 10 mg./kg., and a 3–4-fold increase with 20 mg./kg., of an aqueous solution of hexamethonium bromide injected intraperitoneally. For economy, we used young mice (6–8 weeks old and weighing approximately 20 g.) of homogeneous stock; but older mice were about as sensitive to hexamethonium on a weight basis.

Observational Errors.—With the eyepiece scale used, the possible error in each reading was about half to one unit. Since the value of an increase in pupil diameter involves the difference between two readings, the possible observational error in the estimate would range from about 7% to as much as 50% according as the difference was as large as 15 units (equal to 1.1 mm.) or as small as 4 units (0.3 mm.). This suggests that measuring the total pupil diameter only would give a better estimate of the degree of mydriasis, provided that the initial pupil diameter did not vary in one direction. However, analysis of such results as those given in Table IV shows no appreciable difference whether the increase in pupil size or the total diameter of the pupil was used.

The only serious discrepancy noticed in the measurements of 4 different observers working on different days lay in the time at which the maximum mydriatic effect was obtained; one observer noted it much later (30–40 min.) than the others (10–20 min.). It was then found that the former had been injecting hexamethonium bromide dissolved in 0.9% NaCl, the latter hexamethonium bromide dissolved in distilled water. The mydriatic effects of the two solutions were therefore compared simultaneously on batches of 20 mice. These experiments showed that the maximum response was obtained less rapidly when hexamethonium was given in saline (Fig. 2); nevertheless the difference was smaller than that expected from the difference between observers.

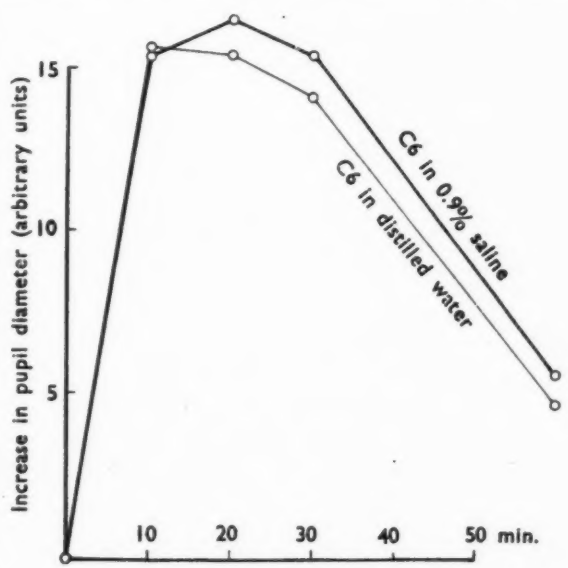

FIG. 2.—Mydriatic effect of hexamethonium bromide (20 mg./kg.) in mice at different times after its intraperitoneal injection. The upper (heavy) curve gives the mean increase for 10 mice given 0.2% hexamethonium dissolved in 0.9% NaCl; the lower curve gives the mean increase for 10 mice given hexamethonium dissolved in distilled water.

Tolerance to Hexamethonium.—As Mohanty (1955) has shown, mice develop considerable tolerance to the mydriatic action of hexamethonium. We have performed experiments similar to his, taking the mean figures for groups of 15 mice given hexamethonium daily (Table III), and some in which the mice were individually identified (Table IV). The latter showed that even by the third day there was a statistically significant

TABLE III

DEVELOPMENT OF TOLERANCE TO THE MYDRIATIC ACTION OF HEXAMETHONIUM

Fifteen mice were used for each experiment. The mice were given one test dose (5 mg./kg.) and two larger doses (each 20 mg./kg.) of hexamethonium bromide each day for 5 days. The mean increases in pupil size given below were measured 15 min. after the intraperitoneal injection of the test dose

Day	Increase in Pupil Size (Arbitrary Units)		Mean Initial Pupil Diameter (Arbitrary Units)
	Mean	S.D.	
1	4·33	2·28	4·33
2	3·87	2·64	4·46
3	2·67	2·41	4·67
4	2·20	1·65	4·60
5	2·00	1·73	4·46
1	4·40	2·30	4·20
2	3·34	1·56	4·93
3	2·20	1·59	4·40
4	1·87	1·31	4·74
5	1·33	1·20	4·60

TABLE IV

DEVELOPMENT OF TOLERANCE TO THE MYDRIATIC ACTION OF HEXAMETHONIUM IN INDIVIDUAL MICE

Hexamethonium bromide (20–40 mg./kg.) was given by intraperitoneal injection 3 times each day for 5 days to 36 individually marked mice from 6 litters. On days 1, 3, and 5, a test dose (5 mg./kg.) was given intraperitoneally before the first large dose of the day. The increase in pupil diameter was recorded 18 min. after the injection of the test dose

Litter No.	Day of Observation	Increase in Pupil Diameter (Arbitrary Units)	
		Bucks	Does
1	1	4 6 5 5 4	3
	3	4 4 3 7 4	1
	5	3 3 3 4 3	1
2	1	3 6 9 3	4 4
	3	2 3 6 2	5 7
	5	1 2 2 2	1 1
3	1	2 3 4	3 4 6
	3	0 3 3	1 3 1
	5	2 0 1	1 2 1
6	1	5 2 11	3 3 7
	3	3 3 8	1 3 2
	5	4 3 7	3 0 1
5	1	2 6	4 5 4 5
	3	2 3	2 6 5 3
	5	0 1	2 1 3 2
4	1	2	2 2 2 4 4
	3	2	0 2 1 1 3
	5	1	0 1 0 1 3

reduction in the response to hexamethonium. As tolerance developed, the decrease in mydriatic effect was roughly proportional to the initial response to hexamethonium, and few mice did not appear to develop tolerance under these conditions (in which the mice were given two loading doses as well as the smaller test dose of hexamethonium each day). On the other hand, the test dose of hexamethonium bromide (10 mg./kg.) could be given several times without the development of a significant degree of tolerance so long as there was an interval of several days between successive doses.

Selection of Mice.—Since it proved difficult to obtain a reliable comparison of different ganglion-blocking agents with small numbers of mice, because of the large variation in the mydriatic response of individual mice, experiments were performed to see whether the variation could be reduced by using litter-mates. An analysis of variance of the results obtained with 36 mice (from 6 litters, each of 6 albino mice, all born within 3 days of one another of parents from a homogeneous population, and containing 18 mice of each sex) showed the within-litter variation to be significantly less ($0.01>P>0.001$) than the between-litter variation. There was also a significant ($P<0.001$) sex difference: the bucks were more sensitive to hexamethonium than were the does. Thus it seems advisable to use mice of the same sex for experiments whose results are to be compared.

In a further experiment, these 36 mice were divided into two equal groups. Each group was treated in turn with hexamethonium at different times on the same day and under the same lighting conditions. Analysis of variance of the results showed both a litter effect and a sex effect significant at the 5% level, and, unexpectedly, an effect due to division of the mice into two groups, significant at the 5% level.

The difficulty of evaluating the factors responsible for variation in the response to hexamethonium became even more evident when an attempt was made to group mice on the basis of their apparent sensitivity to the drug. Three groups of 15–20 mice were treated successively with hexamethonium bromide (20 mg./kg. intraperitoneally), and all those (actually 43%) for which the increase in pupil size differed from the mean by more than 3 units were rejected. When the remaining 30 mice were given a second dose of hexamethonium, the standard deviation was as large as before selection of the mice.

Effects of Other Compounds.—Our chief aim in performing the following experiments was to discover whether the testing of hexamethonium-like compounds for mydriatic activity, which can be done rapidly and cheaply with mice, is likely to give a useful indication as to which compounds possess strong depressor activity. Of the 270 quaternary ammonium compounds tested, 150 were of the type $N—(CH_2)_n—N$, where n = 2, 3, 4, 5, 6, 10, or 12, and the other groups attached to the nitrogen atoms are alkyl or hydroxyalkyl chains or aromatic groups. In another 60 compounds the two nitrogens were linked by such groups as

$—CH_2CH_2OCH_2CH_2—$ and $—CH_2C_6H_4CH_2—$.

The remaining compounds had but one quaternary nitrogen: 22 were of structure $X—N(CH_3)_3$, and the rest differed from hexamethonium still more.

Most of the compounds dilated the mouse pupil to a detectable extent when they were injected intraperitoneally in a dose of 20 or 40 mg./kg., but fewer than twenty possessed activity comparable to that of hexamethonium. With compounds of the type $(CH_2)_n$ $[NRR'R'']_2$ we found high activity only when n was 5 or 6 and the groups R, R′ and R″ together contained no more than 6 carbon atoms—the best cationic heads were such groups as the trimethylammonium, 2-hydroxyethyldimethylammonium, *N* - methylmorpholinium, and *N*-methyl-pyrrolidinium. None of the compounds with a single quaternary ammonium group possessed striking activity. As our main findings agree with those of other workers, such as Wien and his colleagues (1952, 1954), we shall only describe those that indicate defects in this method of assessing ganglion-blocking activity.

Firstly, we should mention that a number of compounds produced mydriasis by mechanisms other than ganglion-blockade. A few were subsequently shown to be atropine-like. With certain other compounds, such as *n*-amyl-trimethylammonium and its near homologues, dilatation of the pupil shortly preceded the death of the animal and seemed to be due, like the accompanying convulsions, to a nicotine-like action. Even when mydriatic activity was apparently due to ganglion-blockade, we sometimes found that the compound had much less depressor activity in the unanaesthetized rabbit than was expected from a large effect on the mouse's pupil (Table II). An outstanding example of this behaviour was provided by the compound 288C53.

Secondly, it is possible that compounds which have little or no mydriatic activity may yet have strong depressor activity. For example, a ganglion-blocking compound which acted almost exclusively on the sympathetic division of the autonomic system would behave in this manner, because the mydriasis produced by hexamethonium-like compounds depends upon blockade of the ciliary ganglion, which lies in the parasympathetic division. We therefore tested a selection of the compounds which had little effect on the mouse pupil on the unanaesthetized rabbit. None of the compounds tested was strongly depressor. However, not much weight can be given to negative results of this type.

Discussion

The important theoretical advantages of determining effects on blood pressure by the Grant ear-capsule technique in rabbits have already been mentioned. In practice, the results obtained with this method have been in good agreement with the results obtained by clinical bio-assay (Table II). The method requires neither expensive apparatus nor much experimental skill. On the other hand, few observations can be made in a 2-hr. session and a satisfactory cross-over experiment takes more than a week to perform. Apart from giving a good indication of the effect of a compound on blood pressure and heart rate, the method enables certain other useful information to be obtained. For example, the fact that the sharp falls of blood pressure obtained with ethoxyethyltrimethylammonium bromide were accompanied by such parasympathomimetic effects as profuse salivation, pupillary constriction, urination and defaecation suggested that this compound is muscarine-like in action—a conclusion reached long ago by Dale (1914). Again, certain of the close chemical relatives of hexamethonium which might otherwise have been of interest because of their depressor activity caused a flaccid paralysis with arrest of respiratory movements. It was noted that when these compounds, and a variety of others, were tested on Bülbring's rat diaphragm preparation, certain of them acted like decamethonium, though more weakly; but this form of activity did not appear to be closely correlated with ability to produce a flaccid paralysis in the rabbit. This is not surprising, for decamethonium and certain related compounds are known to produce paralysis by at least two distinct mechanisms (Zaimis, 1953).

If, as is widely believed, hexamethonium-like compounds lower blood pressure solely by blocking ganglia, the order of activity found in the experiments on rabbits should be roughly the same as that obtained with techniques for measuring ganglion-blocking activity. Some of the discrepancies observed can be seen in Table II. Possibly these may arise through compounds blocking the various ganglia to different degrees. For example, the disparity between the mydriatic and depressor effects of 288C53 may be due to its acting far more powerfully on parasympathetic ganglia than on sympathetic ganglia. However, the recent work of Dontas and Nickerson (1955) suggests that, even with hexamethonium itself, the possibility that the fall of blood pressure is brought about by mechanisms other than ganglion-blockade cannot be ignored. This is one of several reasons why we place especial reliance on the comparison of the depressor effects obtained in the unanaesthetized animal.

There are theoretical objections to using the increase in pupil size as a measure of the potency of hexamethonium-like compounds. Thus mydriasis can be produced by several distinct mechanisms. And even if the action of a compound is confined to ganglia, its action on the ciliary ganglion could conceivably be opposed by an action on the corresponding sympathetic (superior cervical) ganglion. The possibility that blocking actions at these two sites might be distinguished through the use of atropine was investigated by Edge (1953), who gave hexamethonium to atropinized mice to see whether it might produce in them a relative miosis. However, under the conditions of his experiment, hexamethonium had no effect on pupil size. Thirdly, there is the possibility—suggested by much experimental evidence—that the relative blocking activity of hexamethonium analogues varies from one type of ganglion to another.

In view of these objections, we hoped no more of the method than that it would be of value as a "sieving" technique—we wished to discover quickly which of a large number of compounds being tested for useful ganglion-blocking activity should have the earliest attention. From this standpoint, the results obtained were fairly satisfactory. The proportion of compounds which appeared to have considerable ganglion-blocking activity, as judged from the change in pupil size, but which were found on further examination to have little or no activity, was not unduly high—no more than about 1 in 10. All the compounds which other techniques showed to be potent ganglion-blocking agents also dilated the mouse pupil under the conditions described.

The limitations of the Pulewka technique for assessing ganglion-blocking activity are mainly practical. Maximum mydriasis may not occur at the time of measurement. Whereas there is only a moderate variation in the response to a fixed dose of the drug in the one mouse from day to day, there is a considerable variation from one mouse to another. Many observations can be made quickly and easily; one observer can use up to 30 mice at a time—with larger numbers too much time elapses between successive observations on each animal. The accuracy of the method can certainly be increased by using large numbers of mice; but when this is done the expenditure of time is such that the chief practical advantage of the method is lost.

We might add that the constant rate perfusion of the vascularly isolated cat's superior cervical ganglion with a blood substitute (Mohanty, 1955) is our method of choice for accurate comparison of ganglion-blocking compounds.

Summary

1. Practical details are given of two methods used for assessing the depressor activity of compounds tested on account of their chemical resemblance to known ganglion-blocking agents. Theoretical advantages and disadvantages of the methods are discussed.

2. Depressor effects of 38 quaternary ammonium compounds have been compared in chronic cross-over experiments on unanaesthetized rabbits, the blood pressures of which were measured by the Grant ear-capsule technique. The following were estimated to be equipotent intravenous doses of the better-known active compounds: "Ecolid" (2.5 μM), pentolinium (5 μM), Burroughs Wellcome 288C53 (10 μM), "Gaplegin" (20 μM), hexamethonium (25 μM), "Pendiomide" (30 μM).

3. The ganglion-blocking effects of some 300 compounds have been assessed by determining the increase in size of the mouse pupil after intraperitoneal injection of the compound. The order of potency indicated by this technique is appreciably, but not greatly, different from that indicated by the experiments on rabbits.

We are indebted to the following commercial organizations for the supply of numerous compounds and unpublished experimental data: Messrs. May & Baker, Ciba, Burroughs Wellcome, G. D. Searle, Irwin Neisler, the Wm. S. Merrell Co., and the Osterreichische Stickstoffwerke. The expenses of this research were defrayed in part by the Medical Research Council of New Zealand.

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THE EFFECTS OF 5-HYDROXYTRYPTAMINE AND ITS ANTAGONISTS ON TIDAL AIR

BY

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Various reports suggest that 5-hydroxytryptamine (5HT) can cause bronchoconstriction. Thus, Reid and Rand (1952) described a bronchoconstrictor effect in the anaesthetized cat manifested by an increase in intrapleural pressure while respiratory excursions remained normal or decreased. Comroe, van Lingen, Stroud, and Roncoroni (1953) found that pressure-volume curves in anaesthetized cats suggested that large doses of 5HT caused bronchoconstriction. This was explained as mainly a direct constriction of the bronchial muscle, but it also appeared to be partly reflex in origin. Kottegoda and Mott (1955) noted that 5HT increased intratracheal pressure in the vagotomized cat and attributed this to direct bronchoconstriction. A direct effect of 5HT on bronchial muscle, independent of reflexes, has been observed in the perfused lungs of the rabbit (Freyburger, Graham, Rapport, Seay, Govier, Swoap, and Van der Brook, 1952), cat (Gaddum, Hebb, Silver, and Swan, 1953) and guinea-pig (Bhattacharya, 1955). A constrictor effect of 5HT has also been noted in the tracheal chain of the guinea-pig (Freyburger *et al.*, 1952) and cat (Sinha and West, 1953). Furthermore, 5HT administered as an aerosol causes dyspnoea in the guinea-pig, resembling that produced by histamine or acetylcholine (Herxheimer, 1953a, 1953b, and 1955).

As yet, the effect of 5HT on tidal air has not been investigated in the intact and spinal animal. It therefore seemed of interest to make such an investigation with a view to obtaining further information on the mode of action. Another purpose of the study was to investigate whether agents found to be potent antagonists of 5HT in isolated organs *in vitro* also antagonize the bronchoconstriction induced by 5HT *in vivo*.

METHODS

Cats were anaesthetized with a mixture of chloralose (0.05 g./kg.) and urethane (0.5 g./kg.) administered subcutaneously. In the course of some experiments first the vagi were cut and later the spinal cord was sectioned at C 1. Guinea-pigs were anaesthetized by intraperitoneal administration of urethane (1.2 g./kg.). Spinal cats and guinea-pigs were also used, the operation being conducted under ether anaesthesia.

Changes in the distensibility of the lungs at constant pressure were recorded by means of the overflow method of Konzett and Rössler (1940). The lungs were ventilated by a Starling respiration pump driving air into the trachea. The maximum ventilation pressure was kept constant at 8–10 cm. of water. The excess air, which did not enter the trachea, raised a piston carrying a lever which recorded a vertical line on the kymograph. The lever rose during insufflation and fell when insufflation ended. A decrease in the volume of air entering the lungs caused the lever to rise further. Volume calibrations of the ventilation overflow were made in some but not in all experiments.

It may be objected that changes in tidal air, measured by this method, do not distinguish between bronchoconstriction and a displacement of air due to an increase of the volume of blood in the lungs (Barer and Nusser, 1953). It is extremely difficult to settle this question (Barer and Nusser, 1953). The fact that 5HT produced bronchoconstriction in perfused cat lungs without greatly changing the volume of blood in the venous reservoir (Gaddum, Hebb, Silver, and Swan, 1953) may be considered as evidence in favour of the decrease in tidal air being due to a bronchoconstrictor action of 5HT in the intact animal as well. Moreover, in order to produce a reduction in tidal air large volumes of saline (20–40 ml.) had to be injected intravenously (Barer and Nusser, 1953). The rapid injection of 10 ml. saline into the jugular vein had no influence on the tidal air in our experiments. It is improbable that 5HT could elicit such large blood volume changes in the lungs to produce, in this way, a reduction of tidal air. This point will be discussed later.

In some experiments on cats, especially those in which antagonists of 5HT were studied, the thorax was opened and the animal eviscerated. This procedure eliminated changes in intra-thoracic pressure due to variations in abdominal pressure. Moreover, it proved advantageous as 5HT can cause intestinal spasm and bowel evacuation (Erspamer, 1954). In addition, evisceration may be of importance in the

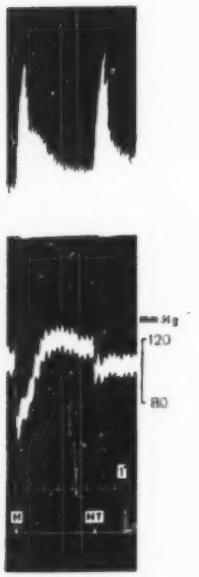

FIG. 1.—Reduction of tidal air induced by histamine (3.4 μg./kg. at H) and by 5-hydroxytryptamine (3.4 μg./kg. at HT) in the anaesthetized cat (2.9 kg.). Blood pressure recorded in the femoral artery. Time in min.

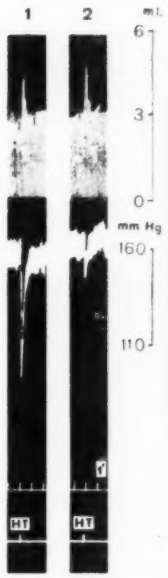

FIG. 3.—Reduction of tidal air induced by 5-hydroxytryptamine (16 μg./kg. at HT) in the anaesthetized cat (3 kg.) before (part 1) and after (part 2) section of both vagi. Blood pressure recorded in the carotid artery. Time in min.

study of 5HT-antagonists as, for instance, radioactive lysergic acid diethylamide is mainly excreted with the bile into the small intestine (Boyd, Rothlin, Bonner, Slater, and Hodge, 1955; Stoll, Rothlin, Rutschmann, and Schalch, 1955).

Systemic blood pressure was measured in the carotid or femoral artery of the cats; in a few experiments on cats, pulmonary arterial pressure was measured, by means of a water manometer, from a branch of one pulmonary artery.

The drugs studied were: 5HT-creatinine sulphate, histamine dihydrochloride, acetylcholine chloride (Roche), D-lysergic acid diethylamide tartrate (LSD), 1-acetyl-D-lysergic acid diethylamide bitartrate, 2-brom-D-lysergic acid diethylamide bitartrate, ergometrine tartrate, atropine sulphate, and 1-methyl-4-amino-*N'*-phenyl-*N'*-(2'-thenyl)-piperidine (Sandosten). All doses refer to the salts.

RESULTS

Effects in the Cat.—5HT produced a reduction of tidal air in 14 anaesthetized cats (Fig. 1) and in the 13 spinal cats. The threshold doses on both preparations ranged from 3–35 μg./kg. A quantitative comparison of the effect on tidal air in the anaesthetized cat and in the spinal cat, utilizing threshold doses, was therefore not profitable. The response to 5HT often increased very markedly during the first 30 min. of the experiment. Small

FIG. 2.—Reduction of tidal air induced by 5-hydroxytryptamine injected intravenously (16 μg./kg. at i.v.) and into the left auricle (32 μg./kg. at i.a.) of the anaesthetized cat (3 kg.). Above: pulmonary artery pressure recorded from a branch of the pulmonary artery by means of a piston recorder (lever adjusted at x). Below: blood pressure recorded in the femoral artery. Time in min.

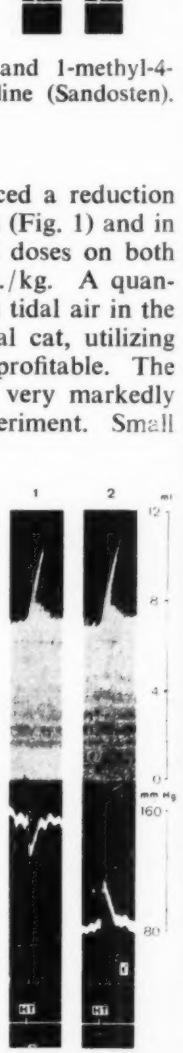

FIG. 4.—Reduction of tidal air induced by 5-hydroxytryptamine (32 μg./kg. at HT) in the anaesthetized and vagotomized cat (3 kg.) before (part 1) and after (part 2) section of the spinal cord at C 1. Blood pressure recorded in the carotid artery. Time in min.

FIG. 5.—Reduction of tidal air induced by 5-hydroxytryptamine (6 μg./kg. at HT) in the anaesthetized guinea-pig (0.65 kg.). The sensitivity to 5-hydroxytryptamine increases in the early part of the experiment.

doses of 5HT usually caused a short-lasting effect on tidal air similar to that of histamine (Fig. 1). 5HT was sometimes as effective as histamine but more often weaker. In good preparations, the effect of small doses of 5HT could be reproduced several times at intervals of 4–10 min.

In order to determine whether the reduction of tidal air produced by 5HT was related to the constrictor action of 5HT on the pulmonary vessels (Ginzel and Kottegoda, 1953; Gaddum *et al.*, 1953) pulmonary arterial pressure was recorded in 3 anaesthetized cats; 5HT was injected intravenously and into the left auricle alternately (Fig. 2). The intravenous injection of 16 μg./kg. 5HT caused a reduction of tidal air, an increase in pulmonary arterial pressure and a decrease in systemic blood pressure. Injection into the left auricle of 32 μg./kg. 5HT caused a reduction of tidal air comparable to that produced by 16 μg./kg. 5HT injected intravenously, a fall in pulmonary arterial pressure and a fall in systemic blood pressure. It may be concluded from this experiment that 5HT decreases tidal air irrespective of whether pulmonary arterial pressure is increased or not. The greater effect on the tidal air after intravenous injection in Fig. 2 may be due to the rise of pulmonary arterial pressure, or to an additional reflex mechanism elicited from receptors in the pulmonary arterial bed. However, in two other experiments in which pulmonary arterial pressure was recorded the reduction of tidal air was the same whether 5HT was injected into the left auricle or given intravenously.

To determine the part played by reflexes in the decrease of tidal air, the effect of 5HT was studied before and after vagotomy in 7 anaesthetized cats. The response to 5HT was not modified by vagotomy in 5 experiments and was slightly decreased in two. Fig. 3 shows an example; the effect of 16 μg./kg. 5HT was much the same before (part 1) and after vagotomy (part 2) in spite of the marked change of the blood pressure response.

The effect of section of the spinal cord at C 1 on the response to 5HT was also studied in 7 anaesthetized vagotomized cats. After cutting the spinal cord, the response to 5HT was not changed in 5 experiments, slightly decreased in one and slightly increased in one. Fig. 4 shows the effect of 32 μg./kg. 5HT on the vagotomized cat (part 1) and on the same animal after cutting the spinal cord at C 1 (part 2). The reduction of tidal air remains much the same in spite of the pronounced difference in blood pressure level and of the divergent effect of 5HT on the blood pressure.

Effects in the Guinea-pig.—5HT diminished tidal air in 9 anaesthetized and in 4 spinal guinea-pigs. The threshold doses on both preparations ranged from 3 to 22 μg./kg. As in cats, the response to 5HT often increased during the first 30 min. of the experiment (Fig. 5). 5HT was either as effective as histamine (Fig. 6) or weaker. Like histamine, 5HT usually produced a short-lasting effect; sometimes, however, its action was prolonged (Fig. 7).

Antagonists of 5HT.—LSD, when given in doses one-third to twice as great as those of 5HT, exerted a specific antagonism towards the effect on tidal air induced by 5HT in 5 anaesthetized and 9 spinal cats and on 8 anaesthetized guinea-pigs (Fig. 6). The reduction in tidal air induced by histamine (Fig. 6), acetylcholine or pilocarpine was not affected by LSD in the smallest doses found to inhibit 5HT.

LSD not only prevented the reduction in tidal air by 5HT; it also abolished a prolonged 5HT-

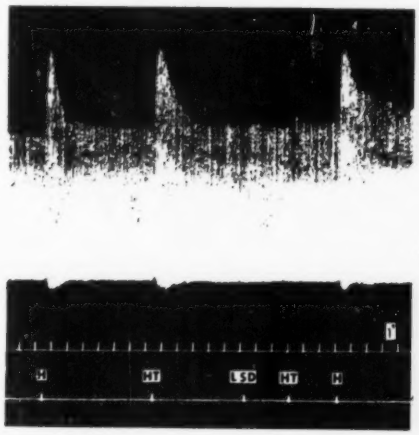

FIG. 6.—The specific antagonism of lysergic acid diethylamide (8.5 μg./kg. at LSD) towards the effect of 5-hydroxytryptamine (17 μg./kg. at HT) on the tidal air of the anaesthetized guinea-pig (0.6 kg.). The reduction in tidal air induced by histamine (17 μg./kg. at H) is not affected by lysergic acid diethylamide.

FIG. 7.—Reduction in tidal air induced by 5-hydroxytryptamine (10 μg./kg. at HT) and increase in tidal air induced by lysergic acid diethylamide (20 μg./kg. at LSD) in the anaesthetized guinea-pig (0.5 kg.). Ergometrine (200 μg./kg. at E) has practically no effect.

effect. An example is shown in Fig. 7; 5HT, 10 μg./kg. caused first a short-lasting and then a long-lasting reduction of tidal air in a guinea-pig. LSD, 20 μg./kg., completely abolished this effect, while 200 μg./kg. ergometrine had practically no effect. A subsequent injection of 5HT again caused a decrease in tidal air which was, however, somewhat less than before LSD.

1-Acetyl-LSD and 2-brom-LSD were as active as LSD in antagonizing the effects of 5HT on the tidal air in 4 anaesthetized and 5 spinal cats. In

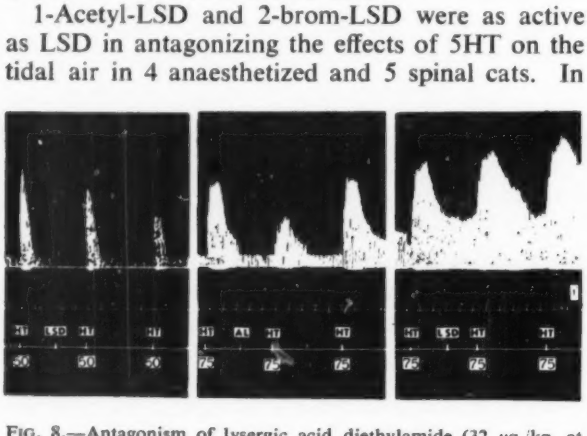

FIG. 8.—Antagonism of lysergic acid diethylamide (32 μg./kg. at LSD) and of 1-acetyl-lysergic acid diethylamide (32 μg./kg. at AL) to the reduction of tidal air by 5-hydroxytryptamine (given at HT; the amount given is indicated in μg./kg.) in the eviscerated spinal cat (3 kg.). The interval between the first part of the experiment and the second is 50 min., that between the second and third part is 12 min.

Fig. 8 the effects of LSD and acetyl-LSD on the spinal cat are compared. In the first part of the experiment 32 μg./kg. LSD diminished the effect of 5HT, the action of LSD being slow to develop but lasting a long time. The amount of 5HT had to be increased from 50 μg./kg. to 75 μg./kg. in order to obtain a response similar to that obtained before LSD. In the second part of the experiment 32 μg./kg. acetyl-LSD produced a short-lasting and partial inhibition of the effect of 5HT. In the third part of the experiment 32 μg./kg. LSD had no effect on the 5HT-induced reduction of tidal air. During this experiment, as in others, some tachyphylaxis developed. A comparison of LSD and 2-brom-LSD in the spinal cat is depicted in Fig. 9. The effect on the tidal air induced by 18 μg./kg. 5HT was partly diminished within 2 min. of giving 18 μg./kg. 2-brom-LSD and even

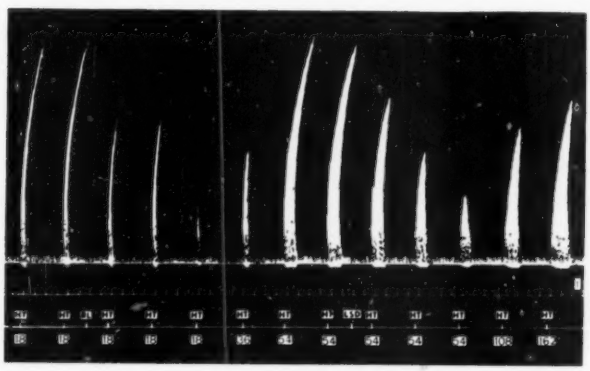

FIG. 9.—Antagonism of 2-brom-lysergic acid diethylamide (18 μg./kg. at BL) and of lysergic acid diethylamide (18 μg./kg. at LSD) to the reduction of tidal air induced by 5-hydroxytryptamine (given at HT; the amount given is indicated in μg./kg.) in the eviscerated spinal cat (2.7 kg.). The amount of 5-hydroxytryptamine given has to be increased during the experiment.

more 10 min. later. By increasing the amount of 5HT it was possible to obtain a response approximately identical with that obtained before 2-brom-LSD. The effect of 54 μg./kg. 5HT—three times the initial amount—was diminished by 18 μg./kg. LSD.

Atropine, in amounts greater than 300 μg./kg. diminished the effect of 5HT on the tidal air in 3 spinal and 1 anaesthetized cats. However, this antagonism was not specific as the effect of acetylcholine on tidal air was inhibited by atropine in far smaller amounts (15–40 μg./kg.) which did not interfere or even enhance the effect of 5HT.

The antihistamine 1-methyl-4-amino-*N'*-phenyl-*N'*-(2'-thenyl)-piperidine ("Sandosten") specifically inhibited the effect of histamine on the tidal air when given in small amounts (80–150 μg./kg.) but did not modify the response to 5HT. When

given in greater amounts (300 μg./kg.) it also diminished the effect of 5HT on the tidal air of 3 spinal cats.

Discussion

5HT decreased the tidal air of cats and guinea-pigs when measured under positive pressure respiration at constant stroke volume. The threshold doses of 5HT to produce this effect varied greatly in both spinal and anaesthetized animals. In general, the reduction of tidal air due to 5HT was short-lasting, resembling the action of histamine. 5HT, however, was usually less potent in cats and guinea-pigs than histamine in reducing the tidal air.

A reduction in tidal air occurs in the anaesthetized cat, whether 5HT is injected intravenously or into the left auricle. In the latter case there is no increase in pulmonary arterial blood pressure. The reduction in tidal air is therefore produced by 5HT independently of its action on the pulmonary arterial pressure.

If the reduction in tidal air were due only or mainly to pulmonary congestion, it would be surprising that the immediate large blood volume changes necessary for an identical reduction in tidal air should occur at such varied blood-pressure levels as are observed in the anaesthetized, vagotomized and spinal cat. Another point in favour of direct action on the bronchial muscles is that histamine had a similar action on the tidal air to 5HT in all experiments in which both substances were given.

Lysergic acid diethylamide (LSD) has been found a potent and specific antagonist of the effect of 5HT in reducing the tidal air of cats and guinea-pigs. This is in agreement with observations on the perfused lungs of cats (Gaddum, Hebb, Silver, and Swan, 1953) and of guinea-pigs (Bhattacharya, 1955), as well as in unanaesthetized guinea-pigs (Herxheimer, 1953b ; 1955). 1-Acetyl-LSD and 2-brom-LSD were about as potent as LSD in cats ; they are also at least as active as LSD in inhibiting the effect of 5HT on the isolated rat uterus and on the isolated rat kidney (Cerletti and Rothlin, 1955 ; Cerletti and Konzett, 1956). Our results demonstrate that, under suitable conditions, those compounds which exhibit an anti-5HT action on plain muscle *in vitro* may act similarly *in vivo*. However, it must be mentioned that LSD, 1-acetyl-LSD and 2-brom-LSD sometimes lose their antagonism to 5HT quite rapidly during an experiment. The reason for this tachyphylactic reaction is not clear. Ergometrine, even in high doses, does not antagonize the effect of 5HT on tidal air.

Atropine also exhibited a remarkable antagonism to the 5HT-effect on tidal air in cats. This is in agreement with the observations of Herxheimer (1953a, 1953b, and 1955), in unanaesthetized guinea-pigs, that atropine effectively antagonized the shock syndrome elicited by an aerosol of 5HT. However, atropine exerted a more specific antagonism towards acetylcholine ; this could be elicited by smaller doses than those required to antagonize the effects of 5HT.

High doses of the antihistamine 1-methyl-4-amino-*N'*-phenyl-*N'*-(2'-thenyl)-piperidine ("Sandosten") also antagonized to some degree the reduction of tidal air induced by 5HT. However, the antihistaminic action of this compound was more specific than its antagonism of 5HT. It may be noted that this antihistamine exerts a pronounced peripheral atropine-like action (Rothlin and Cerletti, 1955). This may be of significance in its antagonism to 5HT, as Herxheimer (1955) found the antihistamine promethazine, which also has marked atropine-like properties, to be an effective antagonist of 5HT, whereas the antihistamine mepyramine, which exerts little atropine-like action, does not protect against 5HT.

Since the reduction of tidal air after 5HT is most probably and mainly due to a direct bronchoconstrictor effect, the antagonistic action of the compounds studied should occur directly on the bronchial muscles and not by interruption of any reflex arc.

Summary

1. 5-Hydroxytryptamine (5HT) produces a decrease of tidal air at constant stroke volume in anaesthetized cats and guinea-pigs, as well as in spinal cats and guinea-pigs. The effect in anaesthetized cats is independent of the action of 5HT on the pulmonary arterial pressure.

2. In anaesthetized cats, the reduction of tidal air due to 5HT is usually identical before and after vagotomy, as well as before and after section of the spinal cord at C 1, suggesting mainly a direct, and not a reflex, action.

3. There are good reasons to suppose that 5HT reduces tidal air by a bronchoconstrictor action.

4. D-Lysergic acid diethylamide, 1-acetyl-D-lysergic acid diethylamide and 2-brom-D-lysergic acid diethylamide specifically antagonize the effect of 5HT on tidal air.

5. Atropine and the antihistamine 1-methyl-4-amino-*N'*-phenyl-*N'*-(2'-thenyl)-piperidine ("Sandosten"), also partly antagonize the action of

5HT on tidal air, but less specifically than they diminish the bronchoconstrictor action of acetylcholine and histamine, respectively.

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CHEMICAL REACTIVATION OF PHOSPHORYLATED HUMAN AND BOVINE TRUE CHOLINESTERASES

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Organophosphates, such as tetraethyl pyrophosphate (TEPP) and di*iso*propyl phosphorofluoridate (DFP), which have a dialkylphosphato group, form with human plasma cholinesterase (also called pseudo- or butyro-cholinesterase) two types of phosphorylated enzyme which possess different stabilities of the enzyme-phosphorus linkage (Hobbiger, 1955). Only phosphorylated enzyme I, which is formed initially, can be readily reactivated by nucleophilic reagents such as nicotinhydroxamic acid methiodide (NHAM). Phosphorylated enzyme I then changes into phosphorylated enzyme II, most probably by transphosphorylation. With DFP or di*iso*propyl *p*-nitrophenylphosphate (D 600) the rate of transphosphorylation is faster than with TEPP or diethyl *p*-nitrophenylphosphate (E 600) and at 37° C. and a *p*H of 7.45 approximately 90% of the inhibited enzyme is converted into phosphorylated enzyme II within 1 hour.

Since the hydrolysis of acetylcholine *in vivo* is mainly accomplished by true cholinesterase (also called aceto-cholinesterase) the formation of a phosphorylated true cholinesterase which cannot be reactivated by nucleophilic reagents could seriously affect the therapeutic usefulness of NHAM and other more potent substances. The experiments on human plasma cholinesterase were therefore extended to human and bovine true cholinesterases which had been inhibited by an organophosphate containing either a diethyl- or di*iso*propylphosphato group and the findings are reported in this paper. In addition to NHAM, ammonium molybdate and pyridine-2-aldoxime methiodide, a potent reactivator of phosphorylated electric eel cholinesterase and phosphorylated human and rat true cholinesterases (Wilson and Ginsburg, 1955; and Childs, Davies, Green, and Rutland, 1955), were used for enzyme reactivation. The results obtained with all these reactivators show that the organophosphates form two types of phosphorylated enzyme with human and bovine true cholinesterases which thus behave like plasma cholinesterase. Only the initial form (phosphorylated enzyme I) can readily be reactivated by the nucleophilic reagents. Transphosphorylation—formation of the truly irreversible phosphorylated enzyme II—occurs both *in vitro* and *in vivo*, and the rate of transphosphorylation is dependent upon *p*H.

METHODS

All experiments were carried out in the Warburg apparatus.

Cholinesterase activity was determined by the manometric technique using 0.01 M-acetylcholine chloride as substrate for human and bovine true cholinesterases and 0.02 M-(±)-acetyl-β-methylcholine chloride as substrate for the true cholinesterases of whole rabbit blood. The procedures adopted for determination of enzyme activity and reactivation were the same as those described in an earlier publication (Hobbiger, 1955).

Except when otherwise stated, the gas phase consisted of 95% N_2+5% CO_2 and a solution containing 0.025 M-$NaHCO_3$, 0.075 M-NaCl, 0.075 M-KCl, 0.04 M-$MgCl_2$ and 0.1% bovine plasma albumin (Armour Laboratories) was used for all dilutions and as the medium during inhibition, reactivation and determination of enzyme activity.

The enzyme preparations used were human red cells which had been twice washed with 0.9% NaCl, purified bovine erythrocyte cholinesterase (Winthrop-Stearns Inc.) and heparinized whole rabbit blood.

The organophosphates used as inhibitors had either a diethyl- or di*iso*propylphosphato group in common; they were:

(a) organophosphates containing a diethylphosphato group:
  tetraethyl pyrophosphate (TEPP),
  diethyl *p*-nitrophenylphosphate (E 600),
  3-(diethoxyphosphinyloxy)-*N*-trimethylanilinium methyl sulphate (Ro 3–0340),
  3-(diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422).

(b) organophosphates containing a di*iso*propylphosphato group:
  di*iso*propyl phosphorofluoridate (DFP),
  di*iso*propyl *p*-nitrophenylphosphate (D 600).

The substances used for reactivation of the inhibited enzyme were:

nicotinhydroxamic acid methiodide (NHAM)*
ammonium molybdate (AM) and
pyridine-2-aldoxime methiodide (P_2AM).

Solutions of these reactivators were freshly made before use. Solutions of NHAM, AM, and trimethylamine (TrMA) were adjusted with NaOH to *p*H 7.45 at which all the experiments were carried out.

Throughout the text the inhibitors and reactivators are referred to by the abbreviations given above.

Results

Interaction of Organophosphates and Reactivators

NHAM, AM and P_2AM react with all the organophosphates which were used for enzyme inhibition to give products which are without anticholinesterase activity. With the reactivator in excess, the inactivation of the organophosphates by reactivators shows the kinetics of a first order reaction (Fig. 1) and no evidence of an equilibrium is detectable. The rate of inactivation of the organophosphates is inversely related to their stability in aqueous solutions, and the differences in potency between the three reactivators are shown in Table I.

The nature of the interaction of organophosphates and reactivators was studied by the manometric technique. Under the same conditions as were used for reactivation of the inhibited enzyme all three reactivators promote the hydrolysis of the organophosphates (Fig. 2). The total amount of acid produced in experiments in which the reaction was allowed to go to completeness was identical with that expected from the appearance of two acidic radicles for each molecule of organophosphate.

Experiments with Human True Cholinesterase

Affinity of Reactivators for Human True Cholinesterase.—In the presence of 0.01 M-acetylcholine chloride only NHAM and P_2AM inhibit the enzyme to any significant extent in concentrations below 10^{-1} M (Table II). The inhibition is readily reversed by dilution and is independent of the sequence in which enzyme, substrate and reactivator are mixed.

Effect of Reactivators on Human True Cholinesterase Inhibited by Organophosphates Containing a Dialkylphosphato Group.—The inhibition of human true cholinesterase by TEPP, Ro 3-0340, Ro 3-0422, or DFP cannot be reversed by addition of substrate, and after a dilution which is sufficient to reduce the organophosphate concentration to ineffective levels

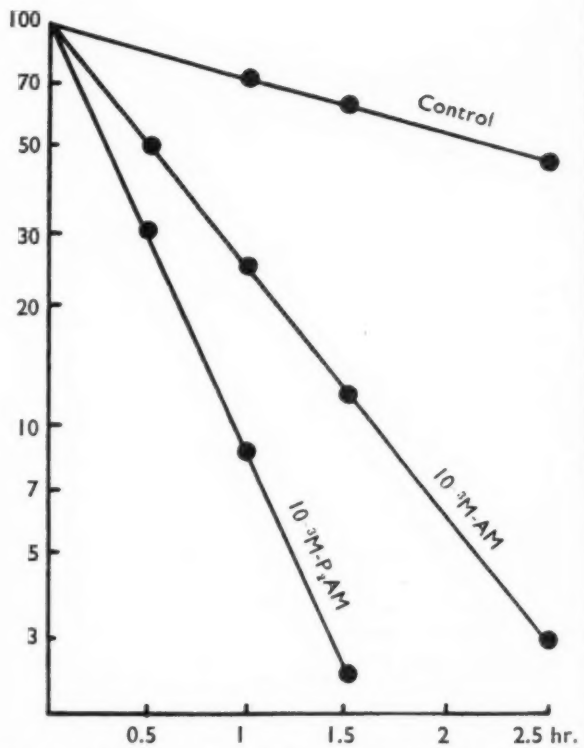

FIG. 1.—Time course of inactivation of 10^{-4}M-TEPP by 10^{-3}M-AM and 10^{-3}M-P_2AM at 37° C. and *p*H 7.45. Experimental conditions as described in legend of Table I. Abscissa: duration of incubation. Ordinate: residual activity in % of the activity of freshly made solutions of TEPP (logarithmic scale).

enzyme recovery never exceeds 5% during the first hour (Burgen and Hobbiger, 1951; and Hobbiger, 1954). This also applies to inhibition produced by E 600 or D 600.

Usually the addition of NHAM, AM or P_2AM to human true cholinesterase, which has been inhibited by one of the above-mentioned six organophosphates, markedly increases enzyme activity

TABLE I

INACTIVATION OF ORGANOPHOSPHATES BY REACTIVATORS AT 37° C. AND *p*H 7.45

All incubations were carried out in the Warburg apparatus using the same medium and gas phase as for reactivation and determination of enzyme activity. The loss in anticholinesterase potency was calculated from the changes in the molar concentration of the organophosphates required for 50 % inhibition of human plasma cholinesterase under standard conditions.

Organophosphate (10^{-4}M)	% Reduction in Activity after 1 Hr. Incubation with						
	–	NHAM		AM		P_2AM	
		10^{-2}M	10^{-3}M	10^{-2}M	10^{-3}M	10^{-2}M	10^{-3}M
TEPP	28	>99	53	>99	75	>99	91
E 600	<1	3	–	6	–	7	–
DFP	18	46	21	>99	53	>99	44
D 600	<1	5	–	6	–	6	–

* In a previous publication (Hobbiger, 1955) NHA was used as the abbreviation; to avoid confusion with nicotinhydroxamic acid (Wilson; 1955, and Wilson, Ginsburg and Meislich, 1955) the abbreviation NHAM is preferred in this paper.

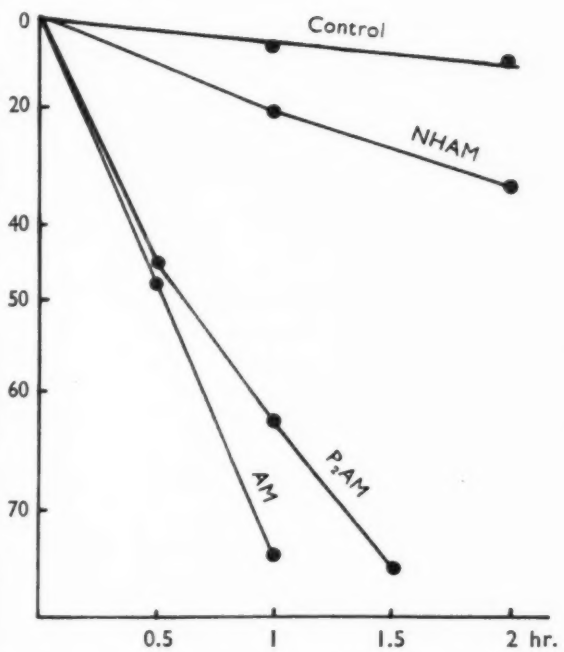

FIG. 2.—Hydrolysis of 2.5 × 10^{-3}M-Ro 3–0422 by reactivators at 37° C. and *p*H 7.45. Ordinate: % hydrolysis of Ro 3–0422 as calculated by the ratio: 100 × amount of CO_2 released/amount of CO_2 that would be released by the liberation of two acidic radicles per molecule Ro 3–0422 (logarithmic scale). Abscissa: time after addition of reactivator (final concentration 2.5 × 10^{-3}M) to Ro 3–0422.

within 1 hour. As with human plasma cholinesterase (Hobbiger, 1955), speed and degree of reactivation of inhibited human true cholinesterase at 37° C. and *p*H 7.45 depend on the type of organophosphate and reactivator, the concentration of free organophosphate, and the duration of enzyme inhibition.

Reactivation after Short Inhibition.—The greatest degree of enzyme reactivation is obtained after short exposures of human true cholinesterase to an organophosphate. The following standard conditions were therefore chosen for studying the effect of the reactivators:

Human red cells were incubated for 20 min. with an organophosphate in a concentration required to produce between 80 and 90% inhibition. The solution containing enzyme plus inhibitor was then diluted with an equal volume of reactivator and 0.3 ml. of this mixture was added to the side-arms of the manometric vessels for further incubation. Enzyme activity was determined by adding the contents of the side-arms to the centre of the manometric vessels which contained 2.7 ml. of substrate dissolved in buffer. The percentage reactivation was calculated from the expression:

$$\frac{100 \times (\text{activity of reactivated enzyme} - \text{activity of inhibited enzyme})}{\text{activity of control enzyme} - \text{activity of inhibited enzyme}}$$

Reactivations up to 75 to 80% were used for evaluation of the time course of reactivation and comparison of the activity of the three reactivators.

Under these conditions the following results were obtained:

If the enzyme is inhibited by TEPP or Ro 3–0422, NHAM in a concentration of 5.10^{-2}M or higher readily restores a considerable amount of enzyme activity within 1 hour and the time course of reactivation closely approaches that of a first order reaction (Fig. 3). The same concentrations of

TABLE II

Reactivator	Molar Concentration Producing 50% Enzyme Inhibition in the Presence of 0·01M-acetylcholine
P_2AM	$3{\cdot}5 \cdot 10^{-3}$
NHAM	$5 \cdot 10^{-2}$
AM	$\gg 10^{-1}$

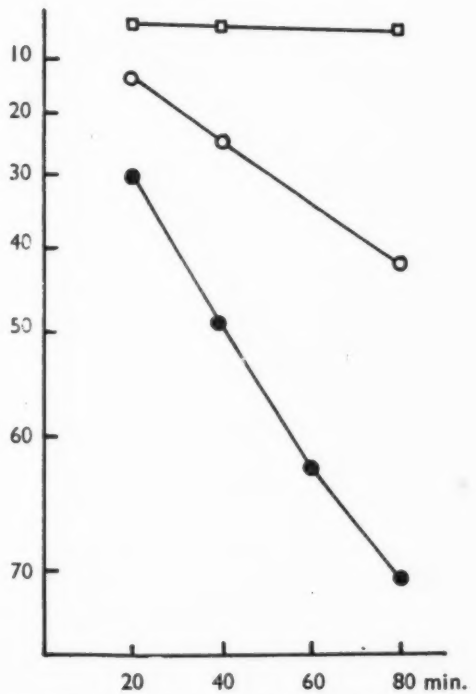

FIG. 3.—Enzyme reactivation by NHAM. The enzyme was first incubated for 20 min. with 2.10^{-7}M-TEPP or 10^{-6}M-DFP. For reactivation equal volumes of reactivator and the mixture of enzyme plus inhibitor were used. ●——●: inhibition by TEPP; reactivator, 1.10^{-1}M-NHAM. ○——○: inhibition by TEPP; reactivator, 5.10^{-2}M-NHAM. □——□: inhibition by DFP; reactivator, 1.10^{-1}M-NHAM. Abscissa: duration of exposure of the inhibited enzyme to NHAM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

NHAM also reduce inhibition by Ro 3–0340 or E 600, but the rate of enzyme reactivation becomes progressively slower with time. NHAM has only a very small effect on the inhibition produced by DFP or D 600, and enzyme recovery amounts to less than 10% during the first hour after addition of 0.1 M-NHAM to the inhibited enzyme (Fig. 3).

AM is more effective than NHAM in restoring enzyme activity after inhibition by TEPP, Ro 3–0422, or DFP, and the rate of reactivation always closely approaches that of a first order reaction (Fig. 4). If the enzyme is inhibited by DFP, the concentration of AM needs to be 22 times higher to obtain the same effect as after inhibition by TEPP or Ro 3–0422. With either Ro 3–0340, E 600 or D 600 as inhibitor, less than 25% of the enzyme activity is restored by AM in concentrations which within 1 hour restore 60% of the activity of an enzyme inhibited by a more labile organophosphate with the same dialkylphosphato group (Fig. 4).

P_2AM is the most potent reactivator, but like AM its effectiveness depends on the organophosphate which is used for enzyme inhibition. With TEPP

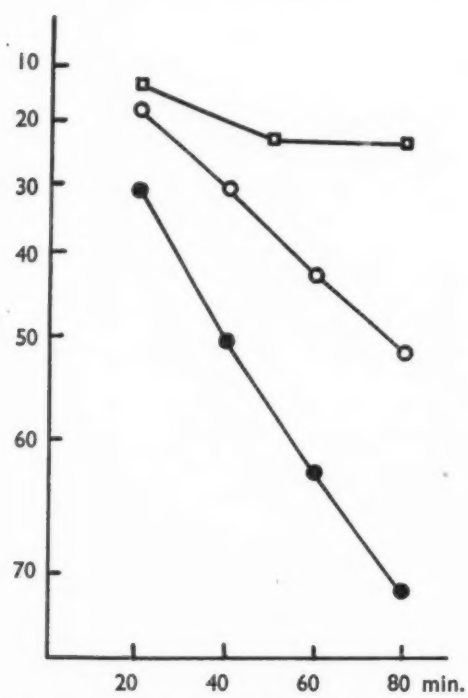

FIG. 4.—Enzyme reactivation by AM. The enzyme was first incubated for 20 min. with 1.10^{-9}M-Ro 3–0422, 5×10^{-9}M-E 600 or 10^{-6}M-DFP. For reactivation equal volumes of reactivator and the mixture of enzyme plus inhibitor were used. ●——●: inhibition by Ro 3–0422; reactivator, 3.10^{-3}M-AM. □——□: inhibition by E 600; reactivator, 3.10^{-3}M-AM. ○——○: inhibition by DFP; reactivator, 4.10^{-3}M-AM. Abscissa: duration of exposure of the inhibited enzyme to AM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

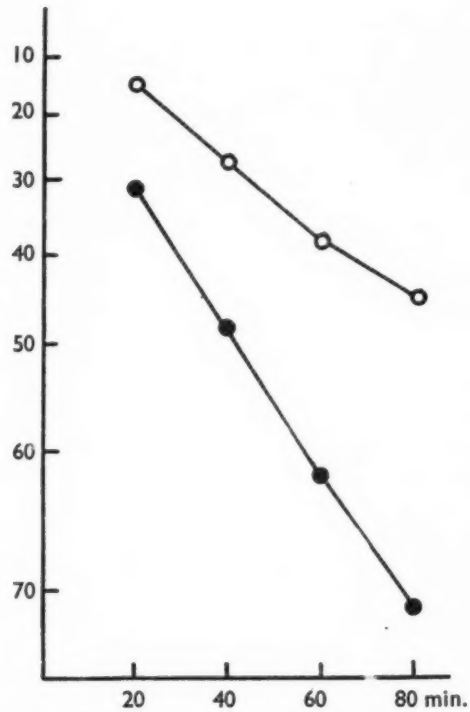

FIG. 5.—Enzyme reactivation by P_2AM: The enzyme was first incubated with 2.10^{-7}M-TEPP for 20 min. Equal volumes of reactivator and the mixture of enzyme plus inhibitor were used for reactivation. ●——●: reactivation by 3.10^{-5}M-P_2AM. ○——○: reactivation by 3.10^{-5}M-P_2AM in the presence of 2.10^{-1}M-TrMA. Abscissa: duration of exposure of the inhibited enzyme to P_2AM before addition of substrate. Ordinate: enzyme activity in % of control (logarithmic scale).

as the inhibitor P_2AM in a concentration of 2.10^{-5}M or higher restores a considerable amount of enzyme activity within 1 hour (Fig. 5). A similar effect is obtained after inhibition by DFP with P_2AM in a concentration of 5.10^{-4}M or higher. The rate of enzyme reactivation approaches that of first order reaction in each instance. Concentrations of P_2AM which reactivate an enzyme inhibited by TEPP are less effective if E 600, Ro 3–0340 or Ro 3–0422 are the inhibitors and the rate of recovery deviates from first order kinetics. Similar results are obtained if one compares the reactivation after inhibition by D 600 with the reactivation after inhibition by DFP.

The differences in the rate of reactivation of enzymes inhibited by organophosphates with a common dialkylphosphato group are the result of interference of free inhibitor with the reactivation process.

If enzyme reactivation is allowed to proceed in the centre of the manometric vessels—which is, however, not possible with NHAM since this reactivator markedly inhibits cholinesterase in the concentra-

TABLE III

MOLAR CONCENTRATION OF REACTIVATOR REQUIRED TO PRODUCE 50% REACTIVATION OF COMPLETELY INHIBITED HUMAN TRUE CHOLINESTERASE IN 60 MIN.

The enzyme is incubated with the inhibitor for 20 min. before reactivation and any interference of free inhibitor with the reactivation process is eliminated by dilution.

Reactivator	Inhibition Produced by an Organophosphate Containing a	
	Diethylphosphato Group	Di*iso*propylphosphato Group
NHAM	$7.5 \cdot 10^{-2}$	$> 10^{-1}$
AM	$2.3 \cdot 10^{-3}$	$5.1 \cdot 10^{-2}$
P_2AM	$2.7 \cdot 10^{-5}$	$6.1 \cdot 10^{-4}$

tions required for reactivation (see Tables II and III) —the free inhibitor is 20 times diluted and thus cannot significantly interfere with the reactivation process. Under such conditions enzyme reactivation shows the characteristics of a first order reaction, and identical results are obtained with equimolar concentrations of a given reactivator as long as organophosphates with the same dialkylphosphato group are used. With sufficiently high concentrations of the reactivator more than 90% of enzyme activity can be restored, and the time required to achieve this is inversely related to the concentration of the reactivating agent.

Table III gives the molar concentrations of the reactivators which in the absence of interference by free organophosphate produce 50% enzyme reactivation within 1 hour.

Effect of Trimethylamine (TrMA) on Enzyme Reactivation.—During reactivation NHAM and P_2AM, but not AM, become attached to the anionic site of an enzyme inhibited by an organophosphate with a diethylphosphato group. This is shown by the effect of TrMA on reactivation. TrMA is a competitive inhibitor of human true cholinesterase with an I 50 of 2.10^{-2}M in the presence of 0.01 M-acetylcholine chloride. When 2.10^{-1} M-TrMA is present during reactivation in the side-arms of the manometric vessels (see conditions described above) the following results are obtained:

After inhibition by TEPP, TrMA slows down the rate of enzyme reactivation by NHAM or P_2AM (Fig. 5). TrMA also cancels out the interference by free inhibitor (Ro 3-0340, Ro 3-0422 or E 600) with the reactivation process by preventing further inhibition after reactivation has taken place. Thus in the presence of TrMA very similar rates of enzyme reactivation are obtained no matter which of the four organophosphates containing a diethylphosphato group is used as the inhibitor.

TrMA does not interfere with enzyme reactivation by AM and it has only a negligible effect on the reactivation of DFP inhibited enzyme by P_2AM.

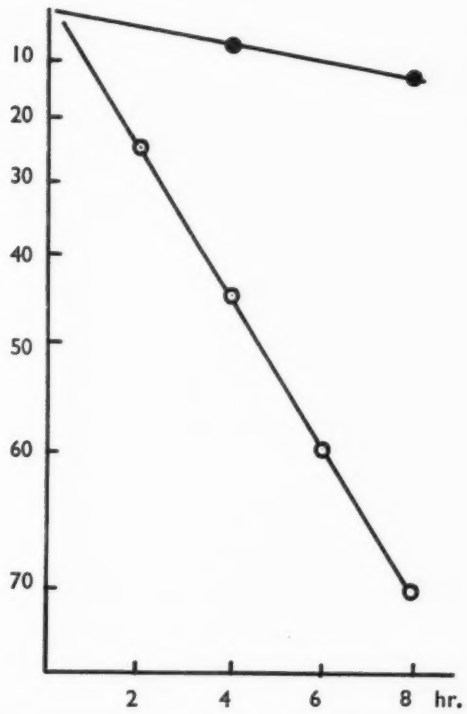

FIG. 6.— Enzyme reactivation after prolonged inhibition. Fresh human red cells were first incubated with the inhibitor. Small samples were taken after various periods of incubation and exposed for 1 hr. to 10^{-2}M-P_2AM. Enzyme reactivation was then determined after a 10-fold dilution. Similar results were obtained when high concentrations of AM were used for enzyme reactivation. ●——●: enzyme inhibited by an organophosphate containing a diethylphosphato group. ○——○: enzyme inhibited by an organophosphate containing a di*iso*propylphosphato group. Abscissa: duration of inhibition before addition of reactivator. Ordinate: % enzyme activity which cannot be restored by the reactivator (logarithmic scale).

Reactivation after Prolonged Inhibition.—The reactivating power of all three reactivators is inversely related to the duration of enzyme inhibition (Fig. 6) and after prolonged inhibition all three nucleophilic reagents are less effective in restoring enzyme activity. Identical findings are obtained with organophosphates which have the same dialkylphosphato group.

The truly irreversible stage of enzyme inhibition, which will be referred to as phosphorylated enzyme II, is not the result of enzyme deterioration during prolonged incubation at 37° C. Enzyme solutions which are kept at 37° C. for 24 hours and are then incubated with an organophosphate for 20 min. can be reactivated to the same extent as solutions of fresh enzyme.

Similarly phosphorylated enzyme II is also not the result of secondary reactions of the organophosphates with cholinesterase. If this were the case its rate of formation should be proportional to the

lability of the ester link of the organophosphate used and to the concentration of free organophosphate. Identical results are, however, obtained using organophosphates which have a common dialkylphosphato group but which differ widely in their stability; and if free inhibitor is removed by addition of 10% rabbit plasma (final concentration) to red cells which have been in contact with TEPP, E 600, Ro 3-0422 or DFP for 20 min. (in a medium of 0.025 M-$NaHCO_3$) the results of reactivation are similar to those observed with controls. Furthermore, a hundredfold increase in the concentration of TEPP or DFP is devoid of any accelerating effect.

Phosphorylated enzyme II thus seems to be the result of transphosphorylation, and its rate of formation will be referred to as "rate of transphosphorylation."

Transphosphorylation is to some extent governed by the incubation medium and proceeds at a faster rate if $NaHCO_3$ is used as the medium instead of a solution which contains $NaHCO_3$ + salt. Results with 95% O_2 + 5% CO_2 did not differ from those with 95% N_2 + 5% CO_2.

All the findings described so far were obtained with fresh red cells as the source of enzyme. The cholinesterase of red cells which have been kept in the deep freeze or over dry ice (in stoppered containers) behaves differently from the cholinesterase of fresh red cells. Whereas fresh red cells lose less than 10% of their cholinesterase activity during 12 hours' incubation at 37° C. under aerobic or anaerobic conditions, the cholinesterase of red cells which have been kept at low temperatures before use is less stable during incubation and shows a greater rate of transphosphorylation under anaerobic conditions (Table IV). Both these effects are far less under aerobic conditions (95% O_2 + 5% CO_2 as gas phase) or under anaerobic conditions (95% N_2 + 5% CO_2 as gas phase) when 10^{-2}M-potassium ferricyanide is present during incubation of the enzyme with an organophosphate. Attempts to make the cholinesterase of fresh red cells behave like the cholinesterase of stored cells by adding a 10^{-2}M concentration of glutathione, cysteine, sodium cyanide or potassium ferrocyanide were unsuccessful.

TABLE IV

EFFECT OF STORAGE AT LOW TEMPERATURES ON THE REVERSIBILITY OF ENZYME INHIBITION

1 hr. contact of the inhibited enzyme with 10^{-2}M-P_2AM was used for reactivation. Inhibition, reactivation and determination of enzyme activity were all carried out in 95% N_2 + 5% CO_2 and with the buffer (described under Methods) as medium.

Source of Enzyme	% Enzyme Activity of Controls after 4 Hr. Incubation	After 4 Hr. Incubation with 2×10^{-7}M-TEPP: % Reactivation
(1) Fresh red cells	98	93
(2) Red cells which have been kept on dry ice for 30 min. before use	81	63
(3) as (2), but after thawing the red cells were kept on dry ice for another 30 min. before use ..	68	24

Experiments with Purified Bovine True Cholinesterase

Purified bovine true cholinesterase which has been inhibited by an organophosphate with a dialkylphosphato group can also be reactivated by NHAM, AM and P_2AM and, on a whole, the results are identical with those obtained with human true cholinesterase. However, one exception was noticed. The reactivation of TEPP-inhibited bovine true cholinesterase by low concentrations of P_2AM, e.g., 3.10^{-5}M, approaches a maximum during the first 20 min. after addition of the reactivator to the inhibited enzyme and then continues only at a low speed. This discrepancy in the behaviour of the two true cholinesterases is the result of interference by free inhibitor. With the red cell preparation it could be shown that no free TEPP was present 30 minutes after the addition of TEPP to the enzyme; this was not so with bovine true cholinesterase. Removal of free TEPP from bovine true cholinesterase by addition of rabbit plasma to the inhibited enzyme before reactivation (Davison, 1954) revealed that the true time course of enzyme reactivation by P_2AM is identical for both enzymes, i.e., reactivation is a first order reaction.

Purified bovine true cholinesterase which has been inhibited for long periods also behaves like human true cholinesterase. On prolonged inhibition two types of phosphorylated enzyme are formed. The time course of transphosphorylation has the characteristics of a first order reaction (Fig. 7) and depends on the medium used during incubation. If Mg^{2+} is replaced by an equivalent amount of Na^+ or if the experiments are carried out in 0.025 M-$NaHCO_3$ transphosphorylation proceeds faster than in the usual buffer medium (see Methods). The removal of free inhibitor (achieved by the addition of rabbit plasma to an enzyme which had been in contact with the inhibitor for 20 min.) does not prevent the formation of phosphorylated enzyme II, and its rate of formation under such conditions is intermediate between that found with 0.025 M-$NaHCO_3$ and 0.025 M-$NaHCO_3$ + salt as incubation media.

Experiments in which 95% O_2 + 5% CO_2 were used as the gas phase instead of 95% N_2 + 5% CO_2 gave identical results, and storage of the enzyme at

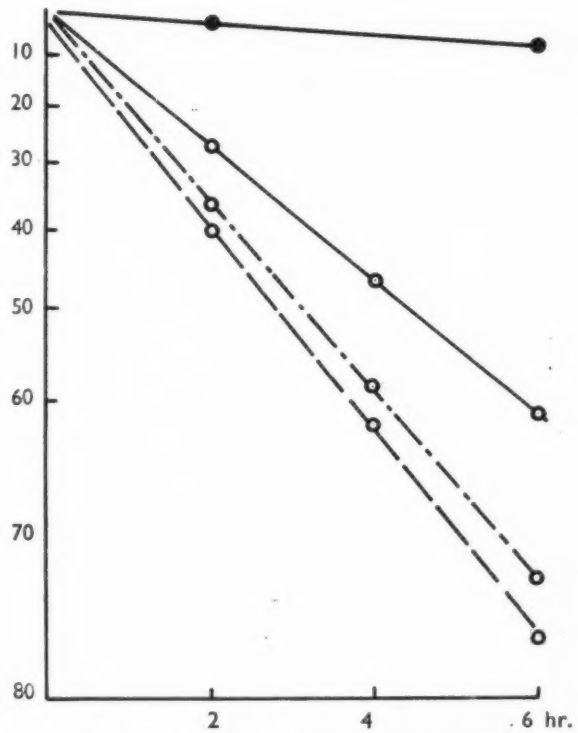

FIG. 7.—Reactivation of purified bovine true cholinesterase after prolonged incubation with organophosphates. Experimental conditions as described in the legend to Fig. 6. Inhibition by TEPP (●——●) or DFP (○——○) using the buffer as medium (described under Methods). ○—.—○: inhibition by DFP using as medium the same buffer but with Mg^{2+} replaced by Na^+. ○——○: inhibition by DFP using 0.025 M-$NaHCO_3$ as medium. Abscissa: duration of inhibition before addition of reactivator. Ordinate: % enzyme activity which cannot be restored by the reactivator (logarithmic scale).

low temperatures before use had no effect on enzyme stability or on the rate of transphosphorylation.

To study the effect of *p*H on transphosphorylation the enzyme was incubated with the inhibitor in solutions containing different concentrations of $NaHCO_3$ while retaining the gas phase of 95% N_2 and 5% CO_2. After a fixed period of incubation the bicarbonate concentration of all solutions was adjusted to 0.025 M and enzyme reactivation was then carried out at *p*H 7.45. The results of these experiments, which are summarized in Table V, show that transphosphorylation is catalysed by acid. An adjustment in the ionic strength of the different media by addition of NaCl had no effect on the results.

TABLE V

EFFECT OF *p*H ON THE RATE OF FORMATION OF PHOSPHORYLATED ENZYME II

Purified bovine true cholinesterase was incubated with 1×10^{-7}M-TEPP for 16 hr. or 1×10^{-6}M-DFP for 2 hr. The inhibited enzyme was then incubated with P_2AM for 1 hr. and enzyme activity was determined after a 10-fold dilution.

*p*H	% Enzyme Activity Restored by 10^{-2}M-P_2AM after Inhibition by	
	TEPP	DFP
8·0	86	75
7·5	77	59
7·0	61	39
6·5	55	33
6·0	52	33

TABLE VI

IN VITRO REACTIVATION OF THE TRUE CHOLINESTERASES OF A RABBIT TREATED WITH DFP *IN VIVO*

Blood samples were taken before and after an i.m. injection of 1 mg. DFP (in arachis oil)/kg. A part of each blood sample was incubated for 1 hr. with 10^{-3}M-P_2AM or 10^{-1}M-AM before addition of substrate. Enzyme activity was determined with 0.02 M-(±)-acetyl-β-methylcholine chloride. All enzyme activities are expressed in % of enzyme activity before the injection of DFP.

Hours After the Injection of 1 mg. DFP/kg.	Enzyme Activity of	
	Untreated Blood	Blood Treated with the Reactivator
0	100*	100
4	36	62
29	21	21
53	35	34
76	53	53

* = 41 μl. CO_2/10 min. Similar results were obtained in 5 other rabbits injected with DFP.

Reactivation of True Cholinesterases of Rabbit's Blood after Inhibition in vivo

The true cholinesterases of rabbit blood behave *in vitro* and *in vivo* like human and bovine true cholinesterases, i.e., they form two types of phosphorylated enzyme. If rabbits are injected intramuscularly with DFP and the activity of the true cholinesterases of whole blood is determined at various intervals afterwards, pre-incubation of the blood samples with P_2AM or AM restores some of the enzyme activity during the early stages of inhibition, but is without any effect when blood samples which have been taken 24 hours after the injection of DFP are treated in the same way (Table VI).

DISCUSSION

The findings reported in this paper indicate that the inhibition of human and bovine true cholinesterases by organophosphates which contain a dialkylphosphato group involves the following stages:

Enzyme + organophosphate → addition complex → phosphorylated enzyme I → phosphorylated enzyme II.

If human and bovine true cholinesterases are exposed to an organophosphate for 20 min. more

than 90% of the enzyme activity can be restored by the nucleophilic reagents NHAM, AM or P_2AM at 37° C., *p*H 7.45, with a bicarbonate-salt solution as medium and in the absence of free organophosphate. Identical rates of enzyme reactivation are obtained with a fixed concentration of a given reactivator if organophosphates which have a common dialkylphosphato group are used as inhibitors and the time course of reactivation has the characteristics of a first order reaction. Since the rates of hydrolysis of organophosphates by nucleophilic reagents vary considerably (Table I), reactivation of the inhibited enzymes must be the result of an action of the nucleophilic reagents on a phosphorylated enzyme.

Trimethylamine reduces the effect of P_2AM and NHAM on an enzyme which has been inhibited by an organophosphate with a diethylphosphato group but has little effect on the reactivation by AM. Thus it seems that the anionic site of the active enzyme surface contributes to the reactivation process by binding and orientating substituted ammonium structures such as P_2AM and NHAM. Such a promoting effect is absent (or negligible) if the organophosphate has a di*iso*propylphosphato group.

In the presence of free organophosphate, enzyme reactivation is governed by the effect of the nucleophilic reagent on phosphorylated and active enzyme and the type and concentration of free organophosphate. Enzyme reactivation is only obtained if the reactivated enzyme is protected by the nucleophilic reagent against further inhibition or if the free organophosphate is hydrolysed by the nucleophilic reagent during enzyme reactivation.

As with human plasma cholinesterase (Hobbiger, 1955), the degree of reactivation of phosphorylated human and bovine true cholinesterases is inversely related to the duration of inhibition. It seems that the dialkylphosphato group becomes initially attached to one reactive group of the esteratic site (to form phosphorylated enzyme I) and then is slowly transferred to another reactive group with which it forms a bond of higher stability that cannot be affected by nucleophilic reagents (phosphorylated enzyme II).

Cohen, Oosterbaan and Warringa (1955) recently obtained from mammalian true cholinesterase, inhibited by DFP, a polypeptide which contained an intact di*iso*propylphosphato group and which gave serine phosphate on acid hydrolysis. Cohen *et al.* concluded from this work that serine is the original point of attack of DFP on the protein. The results presented here make such an interpretation unlikely and support the hypothesis put forward by Wagner-Jauregg and Hackley (1953) that not serine but another amino acid acts as the primary acceptor and forms a labile linkage with the dialkylphosphato group.

The experiments, in which the effect of *p*H on transphosphorylation was investigated, indicate that a group with a *p*H of 7.3 is involved. It is reasonable to assume that the rate of transphosphorylation is determined by the stability of phosphorylated enzyme I. If this is so, the results indicate that a P–N linkage between phosphorus and histidine is initially formed during phosphorylation. The dialkylphosphato group might then be transferred to serine (phosphorylated enzyme II), but the isolation of serine phosphate does not necessarily prove it (Wagner-Jauregg and Hackley, 1953).

The rate of transphosphorylation, which shows the characteristics of a first order reaction, is reduced by Mg^{2+} and depends on the nature of the dialkylphosphato group of the organophosphate. If the organophosphate contains a di*iso*propylphosphato group transphosphorylation is accomplished at a far greater speed than with an organophosphate with a diethylphosphato group (Figs. 6 and 7).

Phosphorylated enzyme II is not the result of enzyme deterioration during prolonged incubation or of secondary reactions of the organophosphates with the enzyme. It has also been demonstrated *in vivo* in rabbits and seems to occur in man. The activity of the true cholinesterase of red cells of a patient with myasthenia gravis who has taken TEPP twice daily ever since he was put on this drug eight years ago (case report No. 2; Burgen, Keele and McAlpine, 1948) was only 3% of the mean activity of true cholinesterase of red cells of 6 control subjects. Incubation of the red cells with 10^{-2} M-P_2AM for 1 hour increased enzyme activity to 7%. Since no case of such a low activity of true cholinesterase of red cells has been reported (for variations of enzyme activity in normal subjects see Davies, 1954), these findings indicate that most of the enzyme was present as phosphorylated enzyme II.

Grob and Harvey (1949) observed that after TEPP the recovery of cholinesterase activity of human red cells *in vivo* was far more rapid during the first 24 to 48 hours than after DFP. After 48 hours the rates of enzyme recovery were similar for both organophosphates—approximately 2%/day of the original enzyme activity. These results can be explained by the transphosphorylation theory, and thus they provide additional indirect evidence for it.

Summary

1. The effects of the nucleophilic reagents nicotinhydroxamic acid methiodide (NHAM), ammonium molybdate (AM) and pyridine-2-aldoxime methiodide (P_2AM) on human and bovine true cholinesterases which have been inhibited by an organophosphate containing a dialkylphosphato group were studied.

2. The results indicate that inhibition of human and bovine true cholinesterases by an organophosphate containing either a diethylphosphato group (TEPP, E 600, Ro 3-0340 or Ro 3-0422) or a di*iso*propylphosphato group (DFP or D 600) is a phosphorylation process which yields two types of phosphorylated enzyme. Only phosphorylated enzyme I, which is formed initially, can be reactivated by the nucleophilic reagents. In the absence of free inhibitor identical rates of enzyme reactivation are obtained if organophosphates with a common dialkylphosphato group are used for inhibition. The time course of reactivation shows the characteristics of a first order reaction.

On prolonged inhibition phosphorylated enzyme I is converted into phosphorylated enzyme II which cannot be reactivated by the nucleophilic reagents. This conversion seems to be the result of a transphosphorylation process which has the characteristics of a first order reaction. If the organophosphate has a di*iso*propylphosphato group transphosphorylation proceeds at a far greater speed than with organophosphates which have a diethylphosphato group.

Transphosphorylation is influenced by the medium used for incubation and occurs also *in vivo*. The *p*H dependence of transphosphorylation indicates that the dialkylphosphato group becomes attached first to the amino group of histidine (phosphorylated enzyme I).

3. All organophosphates are hydrolysed by nucleophilic reagents and the rate of hydrolysis is inversely related to the stability of the organophosphates.

4. Enzyme reactivation in the presence of an effective concentration of free organophosphate is determined by (a) hydrolysis of free organophosphate by the nucleophilic reagent, (b) protection of the enzyme against phosphorylation by the nucleophilic reagent, and (c) reactivation of the phosphorylated enzyme.

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ACTION OF COMPOUND 48/80 ON THE MAST CELLS AND HISTAMINE CONTENT OF GUINEA-PIG TISSUES

BY

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Feldberg and Paton (1951) and Paton (1951) showed that 48/80 is a very potent histamine liberator in dogs and cats. Riley and West (1953) demonstrated a positive correlation between the histamine content and the mast cell population in a variety of tissues, and Riley (1953) showed in experiments on rats that the histamine liberators stilbamidine and (+)-tubocurarine disrupt the mast cells. Mota (1953) and Mota, Beraldo, and Junqueira (1953) observed the disruptive action of 48/80 on rat mast cells both *in vivo* and *in vitro*, and Fawcett (1954, 1955) added further evidence. Norton (1954) observed that the *in vitro* action of 48/80 on rat mast cells is dependent on the concentration of the drug, and developed a method for its assay. These facts support the hypothesis that 48/80 liberates histamine from mast cells.

Not all tissues or species, however, are susceptible to the action of 48/80. Thus Feldberg and Talesnik (1953) have shown that repeated intraperitoneal injections of compound 48/80, which practically deplete the skin and striated muscle histamine in rats, do not reduce the histamine content of the liver, stomach, and duodenum. Later Mota, Beraldo, Ferri, and Junqueira (1954) showed that repeated injections of compound 48/80 in rats depleted the histamine only from those organs in which the histamine is probably of mast cell origin.

The disruptive action of 48/80 on the mast cells was studied by Junqueira and Beiguelman (1955), who suggested that this phenomenon depends upon certain enzymatic systems. Recently, Feinberg and Sternberger (1955) observed that 48/80 failed to produce asthma or emphysema in guinea-pigs, such as usually occurs in anaphylactic or histamine shock, and that the repeated administration of 48/80 caused no desensitization of the animals. They also showed that antihistamines had no protective effect against the drug. In view of these differences between the effects of this drug on the guinea-pig and on the other species (cat, dog, and rat) it was decided to investigate the effect of 48/80 on the histamine and mast cell contents of the guinea-pig.

METHODS

Guinea-pigs of both sexes, 200–500 g., were used. For microscopical observation of the mast cells the tissues were fixed with lead subacetate in acidified alcohol and stained with toluidine blue as previously described (Mota *et al.*, 1954). Since guinea-pig mast cells are rather difficult to preserve the fixative was always injected through the vessels. Lung was fixed by intratracheal or intrabronchial injection. The skin of the lateral region of the head (near the inferior eyelid and snout) was chosen for its richness in mast cells and because it can be easily fixed by injecting the fixative into the carotid artery. Only mesentery was fixed by immersion, stained, and examined as a whole.

Anaesthesia.—When anaesthesia was used the guinea-pigs were injected intraperitoneally with 0.6 ml./100 g. of a mixture containing 10% pentobarbitone sodium and 20% urethane in distilled water.

Histamine Extraction and Assay.—When skin samples were taken the skin was first shaved. The tissues were dried between filter papers, weighed, cut into N-HCl in very small pieces, boiled for 2 or 3 min., neutralized, and assayed on the atropinized guinea-pig ileum by the method of Feldberg and Talesnik (1953). All histamine values are given as base.

RESULTS

Effect of Compound 48/80 on the Lung Mast Cells and Histamine.—Eleven guinea-pigs were used in experiments on the histamine content of the lung. After anaesthesia a cannula was tied into the trachea and then connected to a respiration pump. The thorax was opened and lung samples were collected before, and 2 to 15 minutes after, the intracardiac injection of 0.5 mg. 48/80, in 1 ml. saline. Lung samples were taken alternately from the right and left inferior pulmonary lobes after ligature of the pedicle and then extracted. In another group of three animals the lung samples were fixed and stained for mast cells. Seven out

of the fourteen animals showed respiratory disturbances as shown by diminution in the amplitude of the respiratory movements (four cases), and respiratory stoppage with collapsed lungs (three cases). In the other seven cases there were no observable

TABLE I

HISTAMINE CONTENT (μg./g.) OF GUINEA-PIG LUNG BEFORE AND AFTER THE INJECTION OF COMPOUND 48/80

Guinea-pig	Before	After
1	37·7	43·9
2	36·5	42·8
3	43·5	40·2
4	30·8	28·6
5	59·2	63·4
6	45·0	44·7
7	54·2	56·0
8	32·1	32·2
9	17·5	27·6
10	32·1	30·1
11	28·9	24·1

alterations in the respiratory movements. All the lungs became collapsed and cyanotic. The results of the histamine estimates in these experiments are shown in Table I. No difference could be seen either in the mast cell population or in the histamine content of the lung after the injection of 48/80. This is in contrast to the decrease in the histamine and mast cell content of the sensitized guinea-pig lung after anaphylactic shock (Mota and Vugman, 1956).

Effect on the Blood Histamine.—Twelve guinea-pigs were given intracardiac injections of 0.5 mg. 48/80. Heparinized blood samples were collected by cardiac puncture before and immediately after the death of the animal. Plasma was immediately obtained by centrifugation and assayed for histamine. The results of this experiment are shown in Table II. Although the histamine content of the plasma remained practically unchanged all the injected animals died. Similar experiments with

TABLE II

HISTAMINE CONTENT (μg./ml.) OF GUINEA-PIG PLASMA BEFORE AND AFTER THE INJECTION OF COMPOUND 48/80

Guinea-pig	Before	After
1	0	0·28
2	0·10	0
3	0	0
4	0	0·05
5	0·05	0·05
6	0	0
7	0·22	0·25
8	0·10	0·06
9	0·25	0·25
10	0·10	0·25
11	0·20	0·20
12	0·05	0·05

three rats showed an increase of the plasma histamine ranging from 0.0–0.1 up to 8 to 10 μg./ml.

Effect of Repeated Intraperitoneal Injections of 48/80.—Six guinea-pigs were injected intraperitoneally daily with increasing doses of 48/80 according to the following schedule: 100, 200, 200, 300, 300, 400, 400, 500, 500, 600, 800, 1,000, 1,000, 1,500, and 1,500 μg. No change could be observed in the behaviour of the animals until the dose reached 500 μg. From then on they became uneasy, pawing of the nose and intense scratching being the most frequent signs. Half an hour after the injection of the first 1,500 μg. of 48/80 the animals remained in lateral decubitus, with paralysis of the hindlegs, tremors, and apparent respiratory disturbances. Circulatory changes characterized by ischaemia alternating with hyperaemia were frequently observed in the vessels of the ear. All the signs subsided after three hours. After the second injection of 1,500 μg. 48/80 the animals presented exactly the same signs; one of them died half an

TABLE III

HISTAMINE CONTENT (μg./g.) OF SKIN AND MESENTERY OF CONTROL GUINEA-PIGS, AND AFTER REPEATED INJECTIONS OF COMPOUND 48/80

Guinea-pig	Foreleg Skin		Head Skin		Mesentery	
	Control	Injected	Control	Injected	Control	Injected
1	10·3	7·6	9·2	14·1	10·0	8·7
2	5·7	7·6	11·9	10·4	15·1	16·0
3	4·3	7·0	8·6	11·4	7·7	15·0
4	4·5	8·2	9·5	10·8	16·6	14·2
5	8·7	4·3	9·8	12·1	10·4	22·0
6	8·7	8·7	12·8	11·7	20·0	13·0

hour later, four in 45 min., and the last one in two hours. In all the animals the lungs were collapsed and cyanotic, and five of them had haemorrhagic ulcerations in the gastric mucosa. Six other animals were injected simultaneously with the same volume of saline and were stunned by a blow on the head and bled from the jugular vein.

From both the injected animals and the controls, samples of mesentery and skin (from symmetric sites in the foreleg, paw, and the lateral region of the head) were taken for histamine assay, and samples of mesentery, lung, and skin were fixed for mast cell observation. The results of this experiment are shown in Table III. In spite of repeated injections of large doses of 48/80, no difference could be detected between the histamine and mast cell content of the control and injected animals.

In vitro *Effect of 48/80.*—Since 48/80 has a strong disruptive action on rat mast cells it was decided to test its action directly on the guinea-pig mast

cells. Two guinea-pigs were lightly anaesthetized with ether and bled from the jugular vein. Pieces of mesentery were carefully dissected out and placed in cold isotonic sucrose. This procedure required about five minutes. Two or three pieces were then placed into solutions of 48/80 in isotonic sucrose containing 100, 500, and 1,000 μg./ml. during 15 min. at 37° C., removed, fixed, stained, and examined. In spite of the high concentration of 48/80, and its relatively long and direct contact with the tissues, no modification in the morphology of the mast cells was observed.

Action of Antihistamines.—Twenty guinea-pigs were given intracardiac injections of 2.5 mg./kg. 48/80, ten of them having received intraperitoneally 5 mg. chlorpheniramine hydrogen maleate one hour before. Five of these animals survived, of which three had received the antihistamine. All the other animals displayed spasmodic contractions of the extremities and respiratory embarrassment, and died in 2 to 3 min. At necropsy generalized cyanosis and collapsed lungs were found. The histological examination of the lungs showed atelectasis and mild hyperaemia.

Discussion

Compound 48/80 is an effective histamine liberator in the dog (Paton, 1951; Feldberg and Talesnik, 1953), cat (Paton, 1951; Feldberg and Paton, 1951; and Smith, 1953), rat (Feldberg and Talesnik, 1953; Mota *et al.*, 1954; Riley and West, 1955), and man (Feinberg, Feinberg, Rebhun, and Malkiel, 1954). Our results show that compound 48/80 is not an effective histamine liberator in the guinea-pig, and has no action on its mast cells. These results corroborate those of Feinberg and Sternberger (1955) showing that the release of histamine is not the major toxic action of this drug in the guinea-pig, and those of Feldberg and Mongar (1954), who found 48/80 a poor histamine liberator when guinea-pig lung was perfused with this drug. Although the injection of this drug caused no lung emphysema and no diminution in the lung histamine content, the presence of respiratory disturbances and atelectasis in the lungs suggests that compound 48/80 has some action on the respiratory mechanism. Results of the plasma histamine assays showed that histamine is not liberated in sufficient quantity to raise the plasma histamine level, as it does in the rat (this work) and in the cat and dog (Paton, 1951). Furthermore, the facts that repeated intraperitoneal injections of compound 48/80 do not deplete the skin or mesentery of the guinea-pig of histamine or of mast cells and that the animals do not become refractory to it also suggest that this compound does not have much histamine-releasing action in the guinea-pig. This conclusion is supported by the fact that repeated intraperitoneal injections of compound 48/80 (0.5 mg./kg.) in the guinea-pig before and during the period of sensitization had no effect on anaphylactic shock (Norton, personal communication). In contrast with the rat, the guinea-pig's mast cells and histamine are very resistant to the action of compound 48/80. The dog seems to behave like the rat, since the injection of compound 48/80 promotes a decrease in the liver histamine and mast cells (Mota, unpublished data), and a decrease in the number of mast cells in the tongue (Arvy and Quivy, 1955).

Feldberg and Miles (1953) injected compound 48/80 intravenously in guinea-pigs previously injected with pontamine blue, and observed that different areas of the skin were blued in proportion to their histamine content. Miles and Miles (1952) using the same stain showed intensified blueing of the cutaneous area injected with compound 48/80. This phenomenon was considered to be an effect of the released histamine since it was antagonized by mepyramine. From our own results we think that this histamine-releasing action of compound 48/80 must be very small and localized. However, the intense scratching and the gastric ulcers which we observed in some animals after the injection of this compound do suggest a release of histamine, although we could not detect any decrease in the skin histamine of the animals injected with compound 48/80 compared with the controls. The same thing seems to happen with the mesentery histamine, even when the compound is applied directly. However, a small decrease in the histamine content would be masked by the large individual variations in the results. The fact that in guinea-pig tissues, *in vitro*, compound 48/80 releases as much histamine as the antigen, as observed by Mongar and Schild (1952), might be explained by the dependence of the histamine-releasing mechanism on the experimental conditions (Paton, 1955). In any case it is clear that the main effect of compound 48/80 on the guinea-pig does not depend on the release of histamine or on the disruption of mast cells such as occurs in the rat and dog.

Summary

1. Lethal doses of the histamine liberator 48/80, intravenously administered to guinea-pigs, killed the animals without producing lung emphysema. Antihistamines were ineffective in protecting the animals against this action.

2. Plasma histamine determinations before and after the injection of the compound showed that the histamine level remained practically unchanged. No difference could be detected between the skin and mesentery histamine content of the control and injected animals. Likewise no changes were observed in the mast cells from the lungs, skin, and mesentery. No effect on the mast cells of the mesentery was found when they were directly exposed to the drug.

3. The effect of 48/80 on the guinea-pig is thus not mediated through histamine liberation, and the mast cells and the tissue histamine in this species behave quite differently from those of the rat and dog, being very resistant to the action of compound 48/80.

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THE SPECIFICITY OF BRAIN CHOLINE ACETYLASE

BY

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The relationship between structure and pharmacological action in parasympathomimetic drugs has been extensively studied, notably by Hunt (1915), Hunt and Renshaw (1925, 1929, 1933), Bovet and Bovet-Nitti (1948), and Holton and Ing (1949). These and other studies have shown that few modifications in the acetylcholine molecule are permissible if the same order of pharmacological activity is to be retained. For instance, replacement of one of the methyl groups by ethyl caused little change in activity, but the substitution of *n*-propyl or longer aliphatic groups, or the substitution of more than one methyl by ethyl, led to a pronounced decrease in activity (Holton and Ing, 1949). Substitution of a β-methyl group had little effect on muscarinic activity but almost abolished nicotinic activity (Simonart, 1932). On the other hand α-methyl substitution reduced muscarinic activity without appreciable effect on nicotinic activity. Increase in the separation between the quaternary nitrogen and the ester group to 3 carbons in acetyl-γ-homocholine had little effect on the activity (Hunt, 1915); and finally tertiary, secondary, and primary amino ethyl acetates were much less active than acetylcholine (Stehle, Melville, and Oldham, 1936).

Similar studies on the specificity of the cholinesterases have shown that, although these enzymes are sensitive to the nature of the acyl group, they are extraordinarily unspecific for the alcoholic part of the ester (Adams, 1949), so that even non-basic esters are vigorously split and may sometimes be hydrolysed faster than acetylcholine (e.g., *iso*propenyl acetate—Burgen, unpublished).

Until recently, in default of other evidence, it had been assumed that choline acetylase was specific. Doubt was thrown on this when Banister, Whittaker, and Wijesundera (1953) demonstrated the presence of propionylcholine in ox spleen. Gardiner and Whittaker (1954) and Berman, Wilson, and Nachmansohn (1953) were able to show that acetone powder extracts of pigeon brain and squid ganglia could synthesize propionylcholine or butyrylcholine if propionate or butyrate were added to the incubate in place of acetate. The only suggestion that choline acetylase might also be non-specific for choline, too, was the finding of Korey, Braganza, and Nachmansohn (1951) that choline acetylase from the squid head ganglion was able to acetylate dimethyl ethanolamine as fast as choline, but that ethanolamine, monomethylethanolamine, and diethylethanolamine were not acetylated at all.

The present work was undertaken to study systematically the effects of modification of the choline molecule on the rate of acetylation by choline acetylase and also to attempt to find specific inhibitors of the acetylase.

Since the acetyl esters of many of the bases studied have negligible biological activity it was not possible to use the usual bioassay methods for estimating the rate of acetylation. The most satisfactory solution to this problem was the use of a highly purified preparation of choline acetylase and the measurement of the rate at which the enzyme could transfer acetyl radicles from acetyl-Coenzyme A to the base. This was attained by measuring the rate of deacetylation of acetyl-CoA. The method was applicable to any acetyl acceptor that did not generate a " high energy " acetyl group (it was not applicable for this reason to thiocholine) and did not require modification for the various compounds employed. A further advantage of the method is that it employs the simplest possible acetylation system in which only one enzyme is involved. This eliminates the possibility of indirect inhibition of acetylation which may occur with the usual more complex system in which acetate or citrate must be converted to acetyl-CoA by a phosphokinase system.

For the successful use of this method it is imperative that the enzyme be largely freed of thioesterases. In the presence of these enzymes acetyl-CoA is rapidly split to yield free acetate. When the enzyme is satisfactorily purified there is good agreement between the breakdown of acetyl-CoA and the amount of acetylcholine formed.

METHODS

Preparation of the Brain Enzyme

Acetone Powders.—The brains from 40–80 rats were placed in ice-cold 0.9% saline immediately after removal. The brains were then dried on blotting paper, and, after being freed from the blood vessels and membranes, were homogenized in 200–400 ml. of pure dry acetone at 0° C. in a Waring blender. The acetone was purified by adding solid $KMnO_4$, and after 7–10 days' storage was distilled, dried over anhydrous $CaSO_4$, and then redistilled. The brain suspension was filtered immediately on a coarse sintered glass Buchner funnel and the cake washed three times with ice-cold dry acetone. The powder was spread on filter paper for a few minutes to allow most of the acetone to evaporate and then transferred to a vacuum desiccator over P_2O_5 and evacuated with a water pump for about twenty minutes. The desiccator was stored in the refrigerator. One brain yielded about 0.2 g. of powder.

Extraction and Purification of Choline Acetylase.—The acetone powder was extracted by being ground in a chilled mortar with 13.5 ml./g. of ice-cold extraction medium (0.015 M-K_2HPO_4: KH_2PO_4 *p*H 7.0; 0.058 M-NaCl; 0.001 M-disodium ethylenediamine tetracetate (EDTA); 0.004 M-cysteine). The mixture was allowed to stand at 4° C. for 10 min. and then centrifuged. The supernatant fluid was retained and purified by a variation of the procedure described by Kumagai and Ebashi (1954).

The extract was acidified to *p*H 4.5 with acetic acid and, after overnight storage at 4° C., was centrifuged and the precipitate discarded. The supernatant fluid was adjusted to *p*H 6.8 with $NaHCO_3$ and solid ammonium sulphate added to give a concentration of 16%. After 20 min. the mixture was centrifuged and the residue again discarded. More ammonium sulphate was added to the supernatant fluid to bring the concentration to 36%. After 20 min. storage the mixture was centrifuged and the supernatant fluid discarded. The residue was taken up in 6 ml. of extraction medium and acidified to *p*H 5.0 with acetic acid. After 30 min. at 4° C. the insoluble material was removed by centrifugation. The supernatant fluid was adjusted to *p*H 7.0 with $NaHCO_3$ and dialysed for 1 hr. against a medium containing 0.116 M-NaCl, 0.015 M-K_2HPO_4: KH_2PO_4, 0.001 M-EDTA, and 0.004 M-cysteine. This medium was adjusted to *p*H 7. The enzyme preparation was diluted with the dialysis medium to give a volume of 8 ml./g. of acetone powder. If the preparation was not used immediately it was stored at −10° C.

Acetyl-Coenzyme A

Preparation.—The acetyl-CoA was prepared by the thiolacetate method described by Wilson (1952). Batches of 30 mg. of Coenzyme A (Pabst 85% pure) were acetylated. The acetylation was almost theoretical as shown by the change in extinction at 233 mμ. on alkaline hydrolysis (Lynen, 1953) and by hydroxamic assay.

Determination.—The hydroxamic method of Lipmann and Tuttle (1951) was simplified by using only three solutions: (a), Buffered hydroxylamine, prepared by mixing 25 ml. 4 M-hydroxylamine hydrochloride with 25 ml. 35 M-NaOH and 50 ml. 0.1 M-acetate buffer *p*H 5.4; (b), 10% trichloroacetic acid; (c), 10% $FeCl_3$ in 1.5 N-HCl. Ten min. was allowed for hydroxamate formation after 0.3 ml. of the incubate had been added to 0.1 ml. of reagent (a). We then added 0.1 ml. of (b) and finally 0.1 ml. of (c). The tubes were centrifuged at 3,000 rev./min. for 5 min. and the density read immediately in the Beckman DU spectrophotometer at 540 mμ. Pure acetylthiocholine was used as a standard.

Assay of Acetylcholine and Measurement of Rate of Hydrolysis by Cholinesterase

Acetylcholine was assayed on the guinea-pig ileum. The susceptibility of the acetate esters to hydrolysis by cholinesterase was measured in a Warburg apparatus. The details of the methods used are described by Burgen and Chipman (1951).

Acetylation System

The incubation mixture contained: acetyl-CoA 0.75 μM./ml.; choline or test substrate 11.1 μM./ml.; tetraethylpyrophosphate 0.072 μM./ml.; EDTA 2.2 μM./ml.; K_2HPO_4: KH_2PO_4 *p*H 7; 56 μM./ml.; enzyme equivalent to 30–40 mg. acetone powder/ml. In the inhibition experiments, the test substance was added in a concentration of 11.1 μM./ml.

The mixture was incubated at 36° C. for 30 min. ; the tubes were then placed in an ice bath while aliquots for acetyl-CoA determination were removed. The residue was acidified to *p*H 4–5 for acetylcholine assay.

Substrates

The substrates were either purified from commercially available materials or prepared by standard methods. With the exception of the liquid tertiary compounds which were redistilled all the substances were obtained as pure crystalline salts.

RESULTS

With the purified enzyme the agreement between the formation of acetylcholine and the deacetylation of acetyl-CoA was fairly good, but the purified enzyme still contained some enzymes that could deacetylate acetyl-CoA in the absence of choline or other acetyl acceptors (probably " thioesterases "). To obtain accurate results it was therefore necessary to run a tube containing all the system except choline and take the difference between the amount of acetyl-CoA left after 30 min. incubation of this tube and of the complete system. Table I illustrates a typical experiment. Here the presence of choline resulted in the disappearance of an extra 0.135 μM. acetyl-CoA/ml. At the same time 0.127 μM./ml. acetylcholine was formed. When these precautions were taken the agreement between the two values was within ±20%. The high activity of the enzyme will be noted.

TABLE I

DEACETYLATION OF ACETYL-COENZYME A WITH AND WITHOUT CHOLINE

For composition of acetylation system, see Methods. Incubated for 30 min.

	Acetyl-CoA (μM./ml.)	Acetyl-CoA Utilized (μM./ml.)	Acetyl-choline (μM./ml.)
Complete system, not incubated	0·690	—	0
Complete system ..	0·510	0·135	0·127
Complete system minus choline	0·645	—	0

Acetylcholine formed 3·8 μM./g. powder/30 min.

Test substrates were tested in duplicate in groups of three or four. In each run tubes containing choline and with no substrate were included so that the rate of acetylation could be compared with that of choline. The rate of choline acetylation was 2.5–4.7 μM./g./30 min. in different batches of enzyme.

Table II shows the results obtained on choline and 24 other alkanolamines. Successive replacement of methyl groups by ethyl had little effect and indeed the monoethyl derivative (2) was consistently acetylated at a greater rate than choline. Replacement of the methyl group by longer chains had little effect until *n*-hexyl (7) which was not acetylated. This substance is quite surface active, but the lack of acetylation was not due to enzyme denaturation, as the presence of (7) did not affect choline acetylation. It is interesting that even benzyl (8) or *iso*propyl (9) substitution did not greatly reduce acetylation. Substitution by hydroxyethyl groups was somewhat more inhibitory than ethyl groups, but this decreased activity was only marked in tetraethanolamine (12).

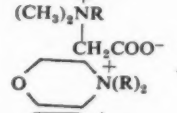
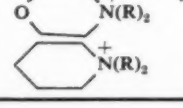
The tertiary, secondary, and primary bases corresponding to choline (13, 14, 15) were all acetylated about one-eighth as rapidly as choline. This change is probably due to the decrease in base strength from the quaternary to the hydrogen bonded lower bases. The differences in base strength between the primary, secondary, and tertiary bases are trivial by comparison. The lack of acetylation of compound 23 may be interpreted in a similar way. This compound, with one methyl group carboxylated giving an electrically neutral betaine, was also devoid of parasympathomimetic activity. The results with the non-quaternary compounds differ from those of Korey *et al.* with the squid enzyme, in which dimethylethanolamine was acetylated as rapidly as choline.

An examination of compounds in which the hydroxyethyl group was modified showed that this was the part of the molecule in which most of the specificity resided. Increase in the hydroxyl-nitrogen separation by one carbon in γ-homocholine (16) abolished acetylation and side chain substitution as in α-methylcholine (18) and β-methylcholine (19) had the same effect. Curiously, increase in the hydroxyl-nitrogen separation to 4 carbons in δ-hydroxybutyltrimethylammonium (17) restored some acetylation.

Three dimeric molecules (20, 21, 22) were tested, and of these the C_{10} member was acetylated at 63% of the rate of choline, but the C_3 and C_5 members were not acetylated at all. Barlow (1955) found that the pharmacological activity of these

TABLE II

ACETYLATION OF ALKANOLAMINES BY RAT BRAIN ENZYME

	Compound R = CH_2CH_2OH	Acetylation
1	$(CH_3)_3N^+R$	100
2	$(CH_3)_2(C_2H_5)N^+R$	143
3	$(CH_3)(C_2H_5)_2N^+R$	102
4	$(C_2H_5)_3N^+R$	95
2	$(C_2H_5)(CH_3)_2N^+R$	143
5	$(n\text{-}C_3H_7)(CH_3)_2N^+R$	74
6	$(n\text{-}C_4H_9)(CH_3)_2N^+R$	67
7	$(n\text{-}C_6H_{13})(CH_3)_2N^+R$	0
8	$(C_6H_5.CH_2.)(CH_3)N^+R$	67
9	$(iso\text{-}C_3H_7)(CH_3)_2N^+R$	67
10	$(CH_3)_2N^+(R)_2$	79
11	$CH_3N^+(R)_3$	63
12	$N^+(R)_4$	16
13	$(CH_3)_2N^+(R)_2$	11
14	$(CH_3.NHR$	15
15	NH_2R	15
16	$(CH_3)_3N^+CH_2R$	0
17	$(CH_3)_3N^+CH_2CH_2R$	29
18	$(CH_3)_3N^+CH(CH_3)CH_2OH$	0
19	$(CH_3)_3N^+CH_2CHOH$ (CH$_3$)	0
20	$RN^+(CH_3)_2$—$(CH_2)_3$	0
21	$RN^+(CH_3)_2$—$(CH_2)_5$—$RN^+(CH_3)_2$	0
22	$RN^+(CH_3)_2$—$(CH_2)_{10}$—$RN^+(CH_3)_2$	63
23	$(CH_3)_2\overset{+}{N}RCH_2COO^-$	0
24		0
25		0

compounds showed a sharp peak at a 10 carbon separation with rapid decline in activity on either side.

Finally two compounds in which the quaternary nitrogen was part of a ring structure (24, 25) were not acetylated.

Inhibition of Choline Acetylation

Inhibition of acetylation was tested by adding an equimolar amount of choline and the test substance to the system and comparing the rate of acetylcholine synthesis with that occurring when choline alone was added. The acetylcholine formation was measured by bioassay. In addition to the compounds listed in Table II, the following were tested: tetramethylammonium, glycine betaine hydrazide, and dicholine ether.

Of the substances that were themselves acetylated only ethyldimethylethanolammonium (2) was an effective competitor. This enzyme appears to acetylate this compound preferentially, but differential assay was inadequate for arriving at an accurate quantitative measure of the relative proportions of acetylcholine and acetoxyethylethyldimethylammonium formed. The most satisfactory method would be chromatographic separation, but this was not attempted. Three other compounds produced a significant degree of inhibition. Carboxymethyldimethylethanolammonium (23) inhibited by 40%, betaine hydrazide 28%, and α-methylcholine 25%. No other substances produced appreciable inhibition. There was never a significantly greater total acetyl ester formation in the presence of a pair of substrates than when either choline or a single substrate was present by itself. This makes it unlikely that any of the substrates are acetylated by an enzyme unable to acetylate choline. We feel justified, therefore, in attributing the acetylations described here to the enzyme commonly called choline acetylase, although this may indeed represent a family of enzymes analogous to the situation with cholinesterases.

Discussion

The present work demonstrates that choline acetylase is by no means specific for choline, but indeed can acetylate many analogues of choline. The effect on enzymic acetylation of replacing the methyl groups of choline is surprisingly trivial in comparison with the marked decrease in pharmacological activity that accompanies the same structural change in acetylcholine. Table III summarizes results obtained in this laboratory and those obtained by Holton and Ing. There is no correlation between the pharmacological activities of the acetic esters and either the acetylation of the alkanolamines or the hydrolysis of the acetic esters. The two enzymic reactions run parallel and so do the two pharmacological properties, but there is no concurrence between the pharmacological and enzymic actions in this series of compounds.

Table III

Summary of Changes Brought About by Replacing the Methyl Groups in Choline and in Acetylcholine

R, R′, R″ in $R'-\overset{R}{\underset{R''}{\overset{+}{N}}}-CH_2CH_2OH$	Acetic Esters			Alkanolamines
	Muscarinic Activity Cat: Fall of B.P.	Nicotinic Activity Frog Rectus	Hydrolysis by ChE	Acetylation by Rat Brain Enzyme
CH_3 CH_3 CH_3	100	100	100	100
CH_3 CH_3 C_2H_5	33*	20*	108*†	143
CH_3 C_2H_5 C_2H_5	0·2*	0·3*	96*†	102
C_2H_5 C_2H_5 C_2H_5	<0·05*	0·02*	94*†	95
CH_3 CH_3 C_3H_7	0·15	0·45	65	74
CH_3 CH_3 *iso*C_3H_7	0·43	0·50	101	67
CH_3 CH_3 C_4H_9	0·08	—	53	67
CH_3 CH_3 $C_6H_5.CH_2$	0·06	—	39	67

* Results from Holton and Ing (1949).
† Source of enzyme dog caudate nucleus. The remaining measurements were made with human red blood cell enzyme.

The failure of any base that is itself acetylated, with the exception of the monoethyl substituted one, to compete effectively with choline, shows that with this exception choline appears to have a smaller dissociation constant for the enzyme-choline complex than the others tested. The Michaelis constant for choline acetylase is not known, but it is very difficult to *abolish* acetylcholine synthesis by quite prolonged dialysis aimed at removing the intrinsic choline. This suggests that the K_m may be quite small. It is therefore likely that the concentration of choline employed in our experiments is very much higher than the level required to saturate the enzyme.

It is of considerable interest to know whether any of the substrates that are acetylated *in vitro* are likely to be acetylated *in vivo* in nerve endings and might thus interfere with normal acetylcholine formation.

The level of choline in the blood varies little and is some 1–2.5 mg./l. (10–25 μM./l.) (Bligh, 1952), but it is by no means certain that the choline concentration in the tissues is similar to that in the blood, or that acetylcholine in tissues is formed from free choline. For instance, Brown and Feldberg (1937) found that the choline content of sympathetic ganglia was 243–520 mg./kg.—some 200 times the blood level—but of this only about 10–20% appeared in the perfusion fluid on prolonged stimulation. Nevertheless very small amounts of choline added to the perfusion fluid increased the

output of acetylcholine in half the preparations tested and also increased the ganglion acetylcholine content. Hutter (1952) has also pointed out that quite small amounts of choline can modify neuromuscular transmission in the cat, and suggests that part of this effect may be connected with acetylcholine synthesis.

These findings suggest that it is possible that other alkanolamines may be able to enter the *in vivo* acetylcholine synthetic pathway. The success of such a substance may well depend on its biological half life and its lack of toxicity. Bligh (1953) has shown that choline disappears from the circulation very rapidly as a result of destruction by choline oxidase. It is desirable therefore that the inhibitor should not be attacked by choline oxidase. Wells (1954) examined the action of choline oxidase on a number of the substrates we are interested in and found that it would not attack non-quaternary alkanolamines or triethyl choline, but readily attacked α and β substituted cholines. Triethylcholine and some of the longer chain aliphatic derivatives might therefore be promising. From the point of view of acute toxicity these are also favourable because of their very low parasympathomimetic activity. Triethylcholine is known to have a low toxicity and will in part replace choline in nutrition (see Sebrell and Harris, 1954).

Summary

1. The activity of purified rat brain choline acetylase was examined on 25 substrates related to choline.

2. The rate of acetylation was little affected by change in the alkyl group substitution, but was abolished or greatly reduced by modification of the hydroxyethyl group or decrease in the basicity of the molecule.

3. Four substances have been demonstrated to inhibit acetylcholine synthesis.

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THE ANTAGONISM BETWEEN 5-HYDROXYTRYPTAMINE AND CERTAIN DERIVATIVES OF LYSERGIC ACID

BY

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The high pharmacological activity of 5-hydroxytryptamine (5-HT) and its wide distribution in the body suggest that it may play a part in normal physiological processes, but this is still a matter for speculation. Very small amounts of 5-HT cause vasoconstriction in the perfused ear of a rabbit, and this tissue was used in the work which first led to the isolation of 5-HT. It has been suggested that an important action of 5-HT may be to modify the responses of tissues to other active substances. The effects on the rabbit's ear of 5-HT combined with various other drugs have been studied by Gaddum and Hameed (1954). The experiments described below are an extension of this work.

METHODS

Rabbits were killed by a blow on the head and bled out. Both ears were removed with a sharp scalpel. The central auricular artery was cleaned and cannulated as described by Page and Green (1948). The ears were perfused at room temperature through a polythene cannula. Two reservoirs were used so that alternative fluids could be perfused by adjusting a two-way stopcock. The ear was fixed on a tilted draining board and perfusate was collected in a glass tube from which it ran to the drop timer (Gaddum and Kwiatkowski, 1938). In the tracings the height of the record indicates the time interval between drops.

The perfusion fluid was that recommended by Page and Green (1948) for the study of vasoconstrictors and had the following composition (g./l.): NaCl 8.2, KCl 0.84, $CaCl_2.2H_2O$ 0.04, $MgCl_2.6H_2O$ 0.06, $NaHCO_3$ 0.4, glucose 1. To each litre was added 10 ml. of phosphate containing 4 parts of M-K_2HPO_4 to 1 part of M-KH_2PO_4.

The ears were more sensitive on the second and third days than on the first day, after being left overnight in the refrigerator.

Sandoz Products Ltd. kindly presented supplies of lysergic acid diethylamide (LSD) and (+)-2-bromolysergic acid diethylamide (Brom LSD). Eli Lilly and Company kindly presented the angiotonin. Other drugs used were ergometrine maleate (B.D.H.) and vasopressin (Pitressin, Parke Davis and Co.), morphine, and methadone.

The doses, which, owing to a misunderstanding and contrary to the usual custom, are given in terms of the weights of the salts, were injected in a uniform volume of 0.1 ml. at regular time intervals. The injections were made slowly through the rubber cap of an injection tube similar to that described by Gaddum and Kwiatkowski (1938) and connected with the polythene cannula. The perfusion fluid entered the air space in this tube in drops, and the rate of injection was regulated so as to keep the size of the drop approximately constant. In this way changes in flow due to changes of pressure in the cannula can be avoided. The "dose-ratio" is the ratio of the dose of 5-HT producing an effect in the presence of the antagonist to the dose producing the same effect in its absence.

RESULTS

The doses were kept small in order to avoid changes in the sensitivity of the preparation. The intervals between injections were 5–10 min. in order to avoid tachyphylaxis, which was particularly liable to occur with 5-HT and tryptamine. When these precautions were taken the sensitivity was reasonably constant and suitable effects were produced by the following doses: adrenaline, 0.5–1 ng.; noradrenaline, 1–2 ng.; 5-HT, 1–10 ng.; tryptamine, 10–100 ng.; pitressin, 1 mU.; and angiotonin, 0.1 unit.

Potentiation

When 5-HT and adrenaline were given together the vasoconstrictor effect was larger than had been expected. A systematic investigation was therefore made of the combined action of various pairs of vasoconstrictor drugs. The drugs used were 5-HT, tryptamine, adrenaline, and noradrenaline. These four drugs can be combined in pairs in 6 possible ways and each of those pairs was studied separately. The two drugs in each pair were first given separately and the concentrations were adjusted until roughly equal effects were produced by 0.1 ml. of solutions of the two drugs. These two equivalent solutions were then mixed in equal volumes, and 0.1 ml. of

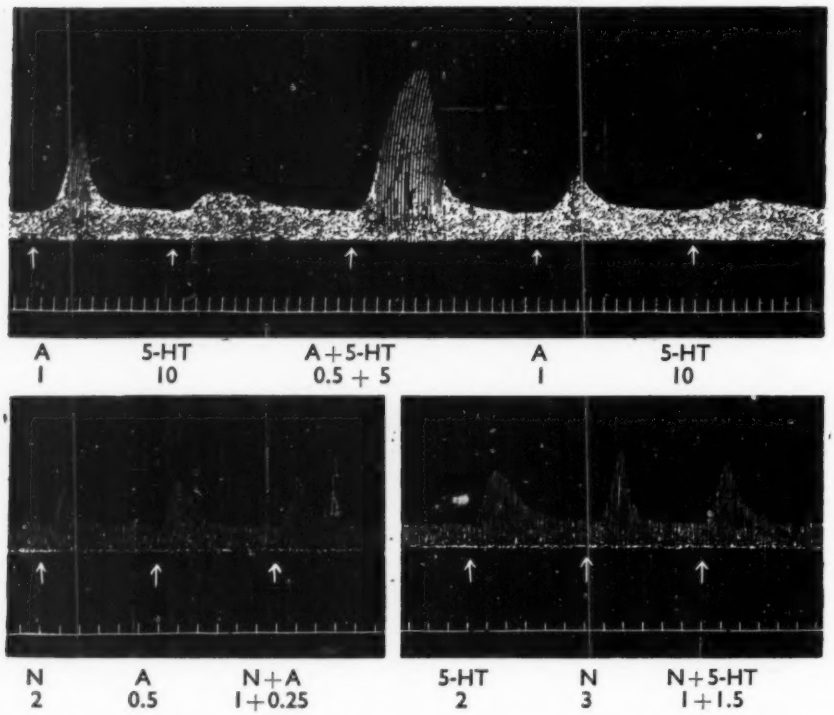

FIG. 1.—Outflow from rabbit's ear. Effects of adrenaline (A), 5-hydroxytryptamine (5-HT), and noradrenaline (N). Doses in ng. Combination of two half-doses of adrenaline and 5-HT shows potentiation. Other pairs of drugs show simple addition.

the mixture, containing half of the original dose of each drug, was injected. When the effect so produced is equal to the original effect, as it would be if the two solutions contained the same drug, the combined action is additive; when the combined effect is larger, there is potentiation. This technique was used by Best, Dale, Dudley, and Thorpe (1927) to show that the depressor effect was potentiated when histamine and choline were injected together into cats.

In the present experiments on the rabbit's ear, potentiation occurred when 5-HT was combined with adrenaline (Fig. 1). This effect was not invariably obtained, but in 13 out of a total of 15 satisfactory experiments with different ears the effect of the combined half-doses was larger than the effect of either of the single doses. The reason for the two failures is not known, but in both cases the initial sensitivity to 5-HT was high, and most of the best results were obtained when it was low. It may be that it is more difficult to sensitize ears which are already sensitive. The sensitivity to adrenaline varied much less.

This potentiation appeared to be exceptional, since none of the other five pairs of drugs showed a similar effect. When tryptamine or noradrenaline was combined with any of the other drugs the effects appeared to be purely additive. When the initial effects were equal the combined effect was equal to them both. When the initial effects were not quite equal the combined effects were greater than one and less than the other (Fig. 1).

Ginzel and Kottegoda (1953) have described potentiation between tryptamine and noradrenaline on the rabbit's ear, but the conditions of their experiments were different. A comparatively large dose of noradrenaline (100 ng.) caused a small vasoconstriction which became larger 30 min. after a large dose of tryptamine. It is thus evident that the potentiation between 5-HT and adrenaline is not unique, but it appears to be especially easy to demonstrate.

LSD

The observation that LSD is a powerful antagonist of 5-HT (Gaddum and Hameed, 1954) has been confirmed. Perfusion of a concentration of 1 μg./l. had a marked effect, increasing for about 2 hr. When low concentrations of LSD are perfused for a short time the response to 5-HT may return after washing (Gaddum and Hameed, 1954). When higher concentrations (20–100 μg./l.) were perfused for longer times, LSD caused an irreversible effect which was not abolished even by washing for 3 hr.

With these higher concentrations the antagonism was unsurmountable (Gaddum, Hameed, Hathway, and Stephens, 1955), so that even large doses of

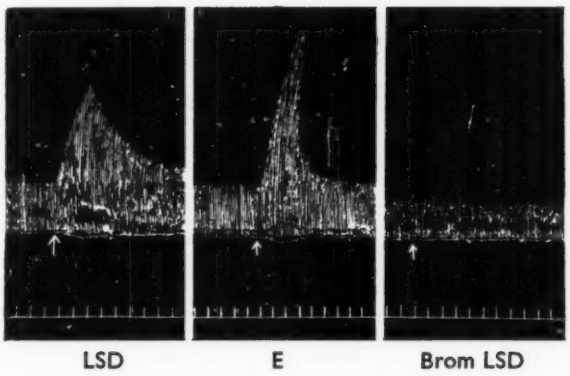

FIG. 2.—Outflow from rabbit's ear. Vasoconstriction due to LSD (1 μg.) and ergometrine, E (0.2 μg.), but not Brom LSD (100 μg.).

5-HT had no action. LSD itself causes vasoconstriction (Ginzel and Kottegoda, 1953), which was sufficient to interfere seriously with the experiment when 100 μg./l. of LSD was present in the perfusion fluid; otherwise even a single small injection (1 μg. in 0.1 ml.) caused a transient vasoconstriction (Fig. 2) with no obvious change in the sensitivity to 5-HT given subsequently.

The effect of tryptamine (100 ng.) was also depressed by LSD in similar concentrations.

The effects of adrenaline (0.5–1 ng.) and noradrenaline (1–2 ng.) were actually increased by LSD (10 μg./l.) so that the recorded height of the rise on the record was often 2–5 times as large as it had been.

Ergometrine

The action of ergometrine resembled that of LSD. It antagonized 5-HT when perfused for 1 hr. in a concentration of 1 μg./l. In a concentration of 10 μg./l. the dose-ratio was about 10, so that doses of 5-HT had to be increased 10 times to reproduce the original effects. Ergometrine appeared to be 2–5 times less active than LSD.

The effect of tryptamine was abolished in much the same way as that of 5-HT.

These results do not agree with conclusions reached by Gaddum and Hameed (1954), and the cause of this discrepancy is unknown, but clear evidence of antagonism has now been consistently obtained in 6 experiments. It is possible that the preparation of ergometrine used by Gaddum and Hameed had become inactive.

These experiments were complicated by the direct vasoconstrictor action of ergometrine itself which appeared to be about 5 times as great as that of LSD. This was clearly shown when 0.2 μg. of ergometrine was injected in 0.1 ml. (Fig. 2). When a concentration of 1 μg./l. was perfused it caused marked vasoconstriction.

The effects of adrenaline and noradrenaline were not definitely affected by ergometrine, even in high concentrations (100–1,000 μg./l.).

Brom LSD

Cerletti and Rothlin (1955) found that Brom LSD was slightly more active than LSD as an antagonist of 5-HT on rat's uterus or perfused rat's kidney. It was also as active an anti-5-HT as LSD in various other tests. It had no action, however, on the brain of man or mouse, and Gaddum and Vogt (1956) found that it failed to cause sham rage in cats such as followed equivalent doses of LSD. These results weaken the evidence for the theory that the actions of LSD on the brain are due to interference with the physiological action of 5-HT on the brain, and it is therefore of interest to study other differences between the actions of these two substances.

Brom LSD was an active antagonist of 5-HT in the rabbit's ear, but it was clearly less active than LSD. It is not easy to give a precise figure for the

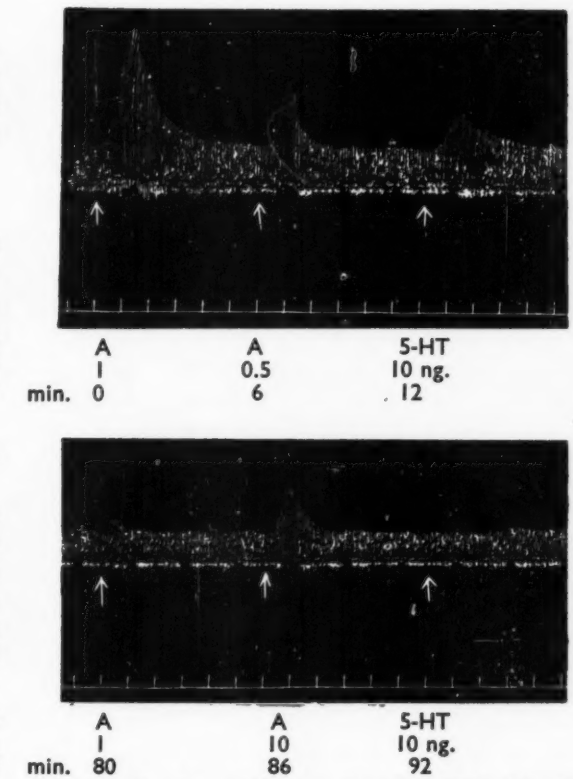

FIG. 3.—Outflow from rabbit's ear. Brom LSD (100 μg./l.). From 50 min. onwards. Doses in ng. Vasoconstrictor effects of both adrenaline (A) and 5-HT diminished.

ratio of the activities, because the action of LSD increased with time for as long as 2 hr., whereas that of Brom LSD was complete sooner, but in experiments where equilibrium conditions seemed to have been reached with both drugs 1 μg./l. of LSD had about the same action (dose ratio 50) as 10 μg./l. of Brom LSD. These results suggest that in these conditions after prolonged perfusion LSD is 10 times as active as Brom LSD.

Brom LSD also differed from both of the other drugs in the following respects:

(1) It never caused vasoconstriction in any dose tested. Thus 100 μg. in a single injection (Fig. 2) had no effect, whereas 1 μg. of LSD caused definite vasoconstriction. A concentration of 2,000 μg./l. was perfused continuously without causing any vasoconstriction.

(2) A concentration of 100 μg./l. reduced the effects of adrenaline and noradrenaline considerably, whereas this same concentration of LSD increased the effects of these drugs. Some antagonism to adrenaline (dose ratio 2–3) was seen after 10 μg./l., and with 1,000 μg./l. the effect was considerable (dose ratio 100–200). It was not possible to test such high concentrations of the other drugs, owing to vasoconstriction.

(3) The antagonistic actions of Brom LSD appeared to develop and disappear more rapidly than those of LSD or ergometrine, and recovery was complete, which was not so with LSD.

(4) The action of large doses of Brom LSD was surmountable by large doses of agonist, but those of large doses of LSD and ergometrine often became unsurmountable.

Morphine

Morphine has been found (Kosterlitz and Robinson, 1955) to have some antagonistic action to 5-HT on the guinea-pig's ileum. No sign of any such effect has been seen in experiments with the rabbit's ear. A concentration of 1 mg./l. had no apparent effect, but when 10 mg./l. was perfused it caused some vasoconstriction and increased the effect of a whole series of vasoconstrictor drugs—adrenaline, noradrenaline, 5-HT, tryptamine, vasopressin, and angiotonin.

Discussion

The potentiation observed when adrenaline and 5-HT were given together was unexpected, and it was surprising that none of the 5 other pairs of drugs showed a similar effect. Results which may be due to the same mechanism were obtained by Lecomte (1953), who injected these drugs intravenously in cats under allobarbitone anaesthesia and found that 5-HT increased and prolonged the effect of adrenaline injected during the next 30 min. Tryptamine did not have this action either in Lecomte's experiments or in those recorded here.

The comparison between LSD, Brom LSD, and ergometrine is of interest because of the actions of LSD and ergometrine on the brain. LSD causes disorders of perception and personality (Stoll, 1947) and sham rage (Gaddum and Vogt, 1956); ergometrine causes sham rage (Brown and Dale, 1935), but psychological changes such as those caused by LSD are not among its recognized effects. Brom LSD appears to have neither effect (Cerletti and Rothlin, 1955; Gaddum and Vogt, 1956), at any rate when tested in equivalent doses. Brom LSD thus differs from the other two drugs both in its failure to cause sham rage and in its failure to cause vasoconstriction in the perfused rabbit's ear. It is possible that these two actions are related to one another.

The activity of Brom LSD relative to LSD was less than that found by Cerletti and Rothlin (1955) in other tissues. This ratio varies according to the conditions, and it is therefore just possible that the failure of Brom LSD to cause psychological changes in man was due to inadequate dosage, but this is not very likely, since it was tested in 20 times the dose found effective when LSD was used.

Summary

1. Potentiation occurred when 5-HT and adrenaline were injected together in the perfused ear of a rabbit.

2. Lysergic acid diethylamide (LSD) and ergometrine in low concentrations (1 μg./l.) both antagonized the vasoconstrictor action of 5-HT or tryptamine, but not that of adrenaline or noradrenaline.

3. In higher concentrations these two drugs have a direct vasoconstrictor action themselves.

4. 2-Bromo-lysergic acid diethylamide in low concentrations (1 μg./l.) also antagonized 5-HT, but it differed from LSD and ergometrine in the following ways:

(*a*) It did not cause vasoconstriction.

(*b*) It antagonized adrenaline and noradrenaline.

(*c*) Its action developed more quickly and recovery was complete on washing.

(*d*) It was always possible to surmount the antagonism with large doses of 5-HT.

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ANTAGONISM BETWEEN CHLORPROMAZINE AND NORADRENALINE IN BLOOD VESSELS OF THE HANDS

BY

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Chlorpromazine (dimethylaminopropyl-*N*-chlorphenothiazine HCl) has a pronounced effect on the circulation in man (Foster, O'Mullane, Gaskell, and Churchill-Davidson, 1954; Duff, McIntyre, and Butler, 1956), causing peripheral vasodilatation as a result of both a central effect and a direct action on the blood vessels (Ginsburg and Duff, 1956). The constrictor response to adrenaline is modified by both intra-arterial and intravenous chlorpromazine (Ginsburg and Duff, 1956); the pressor response to noradrenaline is reduced by intravenous chlorpromazine (Foster *et al.*, 1954). The present investigation is a quantitative study of the effect of intra-arterial chlorpromazine on noradrenaline vasoconstriction in the hands of healthy adults.

METHODS

Twenty-four tests were carried out on ten healthy subjects (nine men and one woman) aged between twenty and thirty-five years. Blood flow in both hands was measured throughout each test by venous occlusion plethysmographs under standard laboratory conditions (Barcroft and Swan, 1953). The flows were calculated and expressed as ml./100 ml. hand volume/min. The general procedure was similar to that previously employed in an analogous study of the action of chlorpromazine and adrenaline (Ginsburg and Duff, 1956). Constrictor responses in the hand to 4-min. infusions of intra-arterial and intravenous noradrenaline were measured first. Noradrenaline was given intra-arterially in doses of 0.125 μg./min. and 0.5 μg./min., and intravenously in a concentration of 10 μg./min. Chlorpromazine was then infused into the brachial artery in a total amount of 1.2 mg., after which the infusions of noradrenaline were replicated in identical order. The course of a typical experiment, as depicted in Fig. 1, consisted first in giving three infusions of noradrenaline, two of which were intra-arterial, the third intravenous; chlorpromazine was then infused intra-arterially, after which the three noradrenaline infusions were repeated. In every test the mean changes in hand blood flow with each infusion were calculated, and analysed by the method described in the previous study of chlorpromazine (Ginsburg and Duff, 1956).

RESULTS

Response to Intra-arterial Noradrenaline Before and After Chlorpromazine

Noradrenaline, 0.125 μg./min.—The effect of chlorpromazine on the constrictor response to noradrenaline was tested in six subjects. A typical experiment is illustrated in Fig. 1. The first infusion of noradrenaline caused a significant reduction (53%) in the mean flow in the test hand. After chlorpromazine (1.2 mg.) had been infused into the brachial artery, the blood flow in the test hand rose considerably; when the infusion of noradrenaline was then repeated, there was only a slight reduction (16%) in flow in the test hand. The results of seven individual tests in which noradrenaline was given intra-arterially before and after the infusion of chlorpromazine are given in Table I. The mean constrictor response to the first infusion of noradrenaline averaged 44% in the seven tests; after the infusion of chlorpromazine the mean response was only 10% (columns [B-E/E]%, Table I). These figures are a measure of the mean decrease in flow in the test hand, expressed as a percentage of the control level of flow in that hand. The percentage constrictor response to noradrenaline was therefore markedly reduced by the infusion of chlorpromazine. A measure of the actual volumetric reduction in flow caused by noradrenaline is obtained from the value of B–E; this may be termed the net constriction. In the seven tests with 0.125 μg./min. noradrenaline the net constriction averaged 3.9 ml. before and 0.7 ml. after chlorpromazine. Thus chlorpromazine reduced the constrictor response to noradrenaline (0.125 μg./min.) both relatively and absolutely.

Noradrenaline, 0.5 μg./min.—The action of chlorpromazine on the constrictor effects of a higher

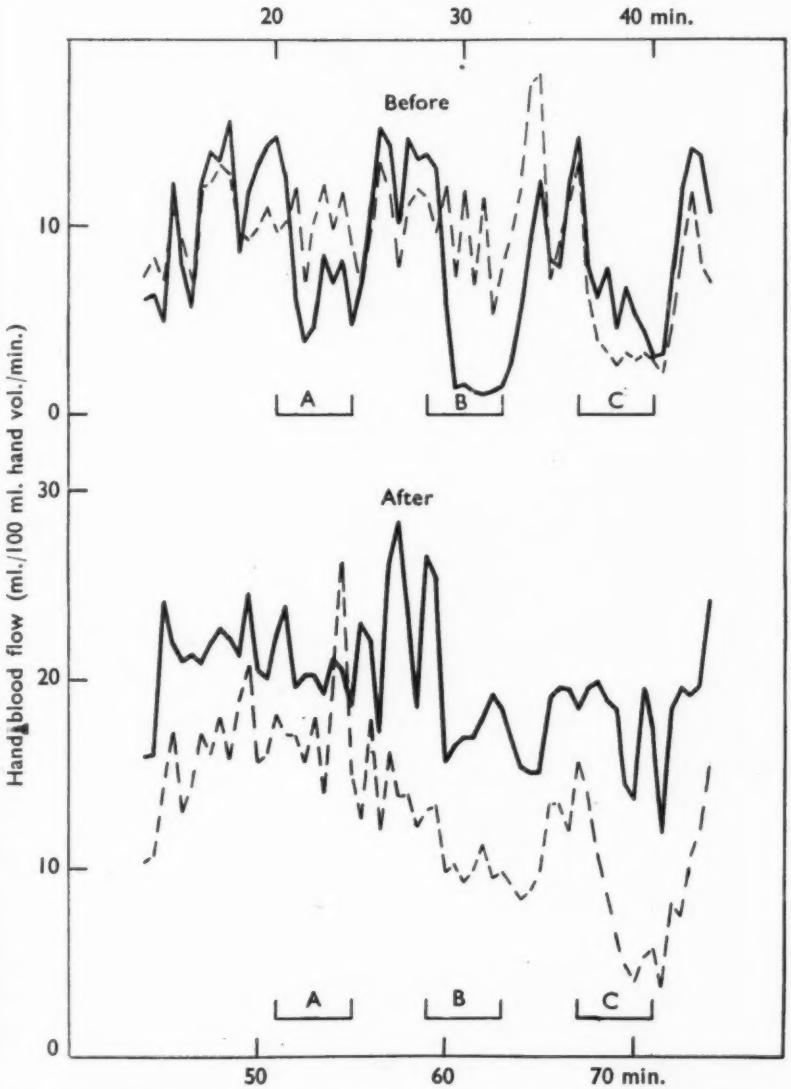

FIG. 1.—Response of hand blood flow in one subject (J. E. ♂) to noradrenaline intra-arterially (A, 0.125 μg./min. for 4 min. and B, 0.5 μg./min. for 4 min.) and intravenously (C, 10 μg./min. for 4 min.) before (upper records) and after (lower records) chlorpromazine (1.2 mg. in 6 min., starting at 44 min. on the record) infused into right brachial artery. Solid lines, right hand; broken lines, left hand.

concentration of noradrenaline (0.5 μg./min.) was examined in ten subjects. The initial constriction was greater with this dose of noradrenaline than with 0.125 μg./min. Thus, in the experiment of Fig. 1, the first infusion of 0.5 μg./min. noradrenaline caused a marked decrease (82%) in flow in the test hand. After the infusion of chlorpromazine, the same dose of noradrenaline caused only a slight decrease (2%) in flow. The detailed results of tests in ten subjects are assembled in Table II. Before chlorpromazine, the percentage reduction in hand blood flow with 0.5 μg./min. noradrenaline averaged 82% in eleven tests; after chlorpromazine, the mean reduction in flow was only 27% (columns [B–E/E]%, Table II). Chlorpromazine clearly caused a marked reduction in noradrenaline vasoconstriction.

The net decrease in flow (B–E) with the initial infusions of 0.5 μg./min. noradrenaline averaged 5.7 ml. whereas after chlorpromazine the net constriction was 3.9 ml. The difference between these means is not significant ($\bar{x}=1.8$, S.E.$=1.4$). The net reduction in flow with 0.5 μg./min. noradrenaline was almost as great after the infusion of chlorpromazine as before it. The percentage constriction was, however, considerably reduced by the chlorpromazine, an effect attributable to the vasodilatation produced by this drug itself. The mean changes in flow are shown diagrammatically in Fig. 2.

Response to Intravenous Noradrenaline Before and After Intra-arterial Chlorpromazine

Noradrenaline was given intravenously in a dose of 10 μg./min. before and after the intra-arterial infusion of chlorpromazine. The first infusion of intravenous noradrenaline reduced the flow in both hands by about the same amount (Fig. 1). After chlorpromazine had been infused into the right brachial artery and the mean flow in that hand had risen, intravenous noradrenaline caused only a slight reduction in flow in the right hand. The second intravenous infusion of noradrenaline caused a marked reduction in flow in the left hand which had not been infused with chlorpromazine. The mean results in six tests with intravenous noradrenaline are summarized in Table III. The mean constrictor response to noradrenaline (10 μg./min.) averaged initially 55% for the right hands and 65% for the left hands (columns [B–A/A]% and [b–a/a]%, Table III). After chlorpromazine had been infused into the right brachial artery, the mean constriction on that side was only 23%; constriction in the left hands, which had not

TABLE I

RESPONSE TO INTRA-ARTERIAL NORADRENALINE (0.125 μG. MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (1.2 MG.) INTO THE BRACHIAL ARTERY

No.	Subject	Before Chlorpromazine					After Chlorpromazine				
		Mean Blood Flow ml./100 ml. Tissue/min.					Mean Blood Flow ml./100 ml. Tissue/min.				
		Test Hand		Control Hand		B–E E%	Test Hand		Control Hand		B–E E%
		A	B	a	b		A	B	a	b	
1	J.E.	12·8	6·4	9·8	10·4	−53	22·2	20·2	17·8	19·3	−16
2	P.J.	6·9	8·5	8·0	12·3	−20	12·7	12·8	16·6	14·9	+12
3	R.D.	7·5	3·6	6·8	6·9	−52	23·9	16·9	16·3	10·2	+13
4	I.D.	8·2	3·8	6·2	6·5	−55	11·7	11·2	12·0	11·6	−01
5	P.J.	13·1	9·9	15·2	13·9	−18	12·7	12·8	16·6	14·9	+12
6	B.B.	3·8	1·2	3·6	2·4	−67	9·4	2·4	4·2	2·9	−63
7	O.R.	7·5	6·1	7·2	10·3	−43	7·8	3·7	6·1	3·9	−26
	Mean	8·2				−44	14·3				−10

A, a, means of six measurements of hand blood flow during 3 min. before noradrenaline in test and control hands respectively; B, b, corresponding means during first 3 min. of noradrenaline period. E=Ab/a.

received chlorpromazine, remained about the same and averaged 55%. The net constriction (B–A) in the test hands with intravenous noradrenaline was 3.9 ml. before, and 4.1 ml. after, chlorpromazine: the difference between those means is not significant ($\bar{x}$=0.2, S.E.=1.4). The infusion of chlorpromazine into the brachial artery thus caused vasodilatation in the corresponding hand and a reduction in the percentage response in that hand to intravenous noradrenaline; chlorpromazine had no appreciable effect on the circulation in the opposite hand.

DISCUSSION

The present study confirms that chlorpromazine causes vasodilatation in the hand, when infused into the brachial artery (Ginsburg and Duff, 1956). This dilator action must therefore be taken into consideration in evaluating other effects of the drug. The results of the experiments designed to assess antagonism to noradrenaline have been analysed to distinguish these actions.

When the results of all the intra-arterial infusions of noradrenaline were assembled, it was found that the initial infusions had reduced the hand blood flow from a mean of 8.0 ml. to 2.9 ml., indicating a net reduction of 5.1 ml. (Fig. 3). After giving chlorpromazine, the blood flow in the infused hands increased to a mean level of 13.5 ml., a rise of almost 70%. The subsequent infusion of noradrenaline caused a reduction in mean flow from the now increased resting level of 13.5 ml. to 10.9 ml., a net decrease of only 2.6 ml. The difference between the mean values for the net vasoconstriction (5.1 ml. before chlorpromazine and 2.6 ml. after) is clearly due to chlorpromazine

TABLE II

RESPONSE TO INTRA-ARTERIAL NORADRENALINE (0.5 μG./MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (1.2 MG.) INTO THE BRACHIAL ARTERY

No.	Subject	Before Chlorpromazine					After Chlorpromazine				
		Mean Blood Flow ml./100 ml. Tissue/min.					Mean Blood Flow ml./100 ml. Tissue/min.				
		Test Hand		Control Hand		B–E/E%	Test Hand		Control Hand		B–E E%
		A	B	a	b		A	B	a	b	
1	O.R.	7·3	0·4	6·1	6·4	−95	9·3	8·7	7·1	8·8	−22
2	B.B.	3·2	0·4	3·0	2·8	−87	9·7	2·3	2·4	2·5	−77
3	J.E.	13·3	2·1	10·7	9·2	−82	23·2	17·0	13·7	10·3	−02
4	R.D.	6·8	0·7	6·4	5·1	−87	27·8	20·2	22·6	16·7	−01
5	I.D.	7·5	2·4	4·8	5·5	−72	10·9	9·7	14·9	9·9	+33
6	P.J.	11·9	1·3	16·9	5·5	−67	17·2	12·4	11·4	8·8	−05
7	D.A.	9·8	2·5	14·4	12·9	−72	12·3	6·6	14·9	16·0	−50
8	C.W.	7·5	0·9	7·8	7·2	−87	14·5	5·8	7·2	6·2	−53
9	R.C.	3·9	0·9	9·9	10·6	−79	19·9	14·2	16·6	16·4	−28
10	N.M.	7·5	1·2	6·3	6·7	−82	16·2	11·8	8·6	10·7	−42
11	D.A.	12·9	0·3	15·7	11·7	−97	12·3	6·6	14·9	16·0	−50
	Mean	8·3		9·3	7·6	−82	15·7		12·2	11·1	−27

A, a, means of six measurements of hand blood flow during 3 min. before noradrenaline in test and control hands respectively; B, b, corresponding means during first 3 min. of noradrenaline period. E=Ab/a.

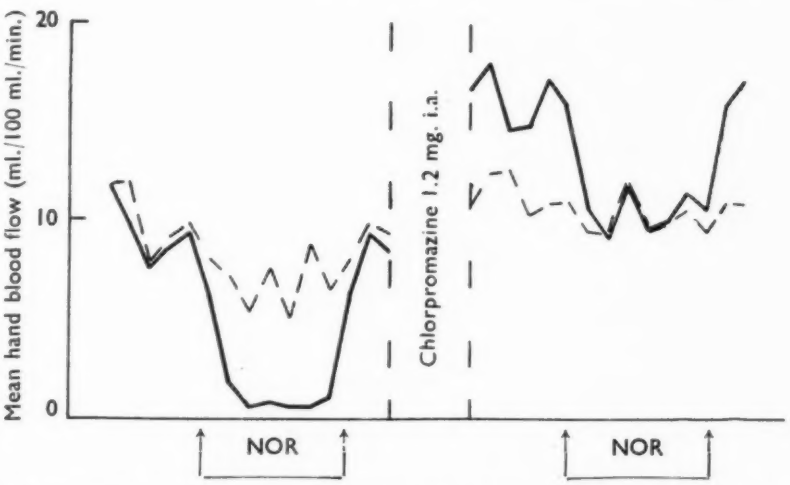

FIG. 2.—Mean blood flow in test hands (solid lines) and control hands (broken lines) during intra-arterial infusions (NOR) of noradrenaline (0.5 μg./min. for 4 min.) before and after intra-arterial chlorpromazine (1.2 mg. in 6 min.).

and presumably reflects a real antagonism to the infused noradrenaline.

When the response to noradrenaline is expressed as the percentage reduction in hand blood flow following its administration, the inhibitory effect of chlorpromazine on noradrenaline vasoconstriction is even greater. Thus the mean percentage vasoconstriction caused by noradrenaline was 82% initially and only 27% after chlorpromazine. The resting level of flow was, of course, higher after the infusion of chlorpromazine (Table II and Fig. 2). The reduction of noradrenaline vasoconstriction by chlorpromazine must therefore be partly attributed to the direct dilator action of chlorpromazine on the blood vessels.

There are several possible explanations for this direct dilator action. It might be due to a blocking of sympathetic constrictor impulses at nerve endings, or to an action on circulating constrictor substances; alternatively the drug might act directly on arteriolar muscle. The extent to which the dilator effect of chlorpromazine depends upon its antagonism to noradrenaline cannot be determined until all the factors responsible for normal vascular tone have been evaluated. The evidence that noradrenaline is intimately concerned in sympathetic transmission favours the possibility that the vasodilatation from chlorpromazine involves an action on sympathetic nerve endings in the vessel walls.

The pressor effect of noradrenaline is reduced by systemic chlorpromazine in animals (Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet, 1953; Kopera and Armitage, 1954) and man (Foster *et al.*, 1954). But this action is comparatively slight, and noradrenaline is clinically effective in the treatment of prolonged hypotension after systemic chlorpromazine (Preston and Wishart, 1954). It is therefore reasonable to infer that the inhibitory effect of chlorpromazine on noradrenaline vasoconstriction is less marked in vascular beds other than those of the hands.

Chlorpromazine has little or no specific action in man against injected adrenaline (Ginsburg and Duff, 1956); the reduction in the constrictor response to adrenaline could be wholly accounted for by the vasodilatation produced by the chlorpromazine. It would therefore appear that in the vessels of the hand chlorpromazine is more effective against noradrenaline than against adrenaline. In laboratory animals, however, chlorpromazine reduces the pressor action of adrenaline to a much

TABLE III

RESPONSE TO INTRAVENOUS NORADRENALINE (10 μG./MIN.) BEFORE AND AFTER INFUSING CHLORPROMAZINE (1.2 MG.) INTO THE BRACHIAL ARTERY

No.	Subject	Before Chlorpromazine Mean Blood Flow ml./100 ml. Tissue/min.						After Chlorpromazine Mean Blood Flow ml./100 ml. Tissue/min.					
		Test Hand		Control Hand		B–A/A%	b–a/a%	Test Hand		Control Hand		B–A/A%	b–a/a%
		A	B	a	b			A	B	a	b		
1	J.E.	10·6	5·9	10·9	3·2	−44	−71	18·5	17·7	12·9	6·7	−04	−48
2	R.D.	8·4	4·1	9·2	3·2	−51	−65	20·2	16·3	11·2	6·8	−19	−39
3	R.C.	4·4	1·9	12·3	4·9	−57	−60	18·5	14·4	19·5	6·9	−22	−65
4	R.C.	3·8	1·8	9·9	4·3	−53	−57	24·6	18·3	21·4	9·0	−26	−58
5	N.M.	8·2	3·5	8·7	3·0	−57	−66	12·8	9·3	5·2	2·2	−27	−58
6	N.M.	7·5	2·5	6·8	2·0	−67	−71	15·2	9·1	9·6	2·8	−46	−60
	Mean					−55	−65					−23	−55

A, a, means of six measurements of hand blood flow during 3 min. before noradrenaline in test and control hands respectively; B, b, corresponding means during first 3 min. of noradrenaline period.

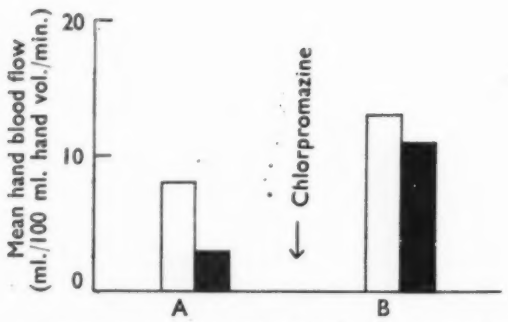

FIG. 3.—Mean blood flow in test hands before (open rectangles) and during (solid rectangles) intra-arterial infusions of noradrenaline (0.125 μg./min. and 0.5 μg./min. for 4 min.) before (A) and after (B) intra-arterial chlorpromazine (1.2 mg. in 6 min.).

greater extent than that of noradrenaline (Courvoisier *et al.*, 1953; Kopera and Armitage, 1954). These differences are less likely to be due to a variation in species than to a different action of the drug on various parts of the vascular tree. Of even greater interest, perhaps, is the curious difference in the effect of chlorpromazine on the differential response of the same vascular bed to adrenaline and noradrenaline.

SUMMARY

1. The effects of intra-arterial chlorpromazine on noradrenaline vasoconstriction in the hands of healthy adults have been assessed by venous occlusion plethysmography.

2. The infusion of chlorpromazine into the brachial artery caused a marked reduction in the constrictor response in the hand to noradrenaline. This effect has been shown to be largely attributable to the direct vasodilator action of chlorpromazine, but partly also to a specific antagonism to infused noradrenaline.

3. Some implications of these findings are briefly discussed.

The authors have pleasure in expressing their indebtedness to Professor H. Barcroft for constant encouragement advice, and valuable criticism; to colleagues and medical students who volunteered as subjects; and to Mr. G. T. Hales and Miss R. Palmer for efficient technical assistance. Certain expenses were defrayed by a grant from the Endowment Fund of St. Thomas's Hospital. Chlorpromazine was supplied by May & Baker, Ltd.; noradrenaline (Levophed) was supplied by Bayer Products, Ltd.

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Brit. J. Pharmacol. (1956), **11**, 323.

OCCURRENCE OF A SUBSTANCE P-LIKE POLYPEPTIDE IN FISH INTESTINE AND BRAIN

BY

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(RECEIVED MAY 18, 1956)

Substance P has been found in relatively high amounts in the intestine and brain of various animals, such as the monkey, horse, ox, pig, sheep, dog, cat, rabbit, and man (Euler and Gaddum, 1931; Douglas, Feldberg, Paton, and Schachter, 1951; Ehrenpreis and Pernow, 1952; Pernow, 1953, 1955; and others). It thus appears to be a regular constituent of these organs in mammals. No definite data are available, however, from other phyla, and it therefore seemed of interest to determine whether or not this highly active polypeptide also occurs in poikilothermic animals.

Substance P may be salted out from crude aqueous extracts by ammonium sulphate (Euler, 1936a, b; Pernow, 1953). A convenient and efficient method of purification, based on adsorption chromatography on aluminium oxide, has been described by Pernow (1953). The present paper reports some experiments made on purified extracts from the intestine and brain of fish.

METHODS

Extracts were prepared from the intestine of freshly caught cod (*Gadus calarias* and *G. morrhua*) and dogfish (*Squalus acanthias*). After removal of their contents the organs were immediately cut into small pieces, heated to boiling in 2 volumes of water, and sulphuric acid added to a final *p*H of 4. After 10 min. boiling the extract was filtered. To the cool filtrate ammonium sulphate was added to a final concentration of 2/3 saturation and the solution left overnight at 5° C. The precipitate was filtered off and pressed nearly dry between filter papers.

The crude extracts were purified according to Pernow (1953). The dry ammonium sulphate precipitate was dissolved in 4 parts of water, and methanol slowly added during continuous stirring to a final concentration of 70%. The voluminous precipitate contained only little activity and was discarded. The 70% methanol extract was run through a column of 5–12 g. aluminium oxide (British Drug Houses) at a rate of 0.5–1 ml./min. The column was washed with 70% methanol, followed by 10–20 ml. of decreasing concentrations of methanol (60%, 50%, etc.) and finally distilled water.

After evaporation of the methanol *in vacuo* the eluates were assayed on a strip of isolated guinea-pig intestine (Pernow, 1951) in a 3 ml. bath of oxygenated Tyrode solution at 38° C., containing atropine sulphate and an antihistamine ("Lergigan," Recip) each in a concentration of 1 : 2.5 million. A purified preparation of Substance P obtained from beef intestine, kindly placed at our disposal by Dr. B. Pernow, was used as standard, and the activity expressed in the P unit previously used in our laboratory. The extracts were also tested on rabbit isolated intestine, and on the blood pressure of the cat under pentobarbitone anaesthesia and of the rabbit under urethane anaesthesia.

The polypeptide nature of the active substance was checked by incubation with pure trypsin ("Tripur," Novo).

RESULTS

Preliminary tests showed that ammonium sulphate precipitates of aqueous extracts of cod and dogfish intestine and cod brain had the typical actions of Substance P, corresponding approximately to 4–6 standard units/g. of fresh organ, when tested on the guinea-pig isolated intestine, treated with atropine and lergigan.

Cod Intestine

Purification.—A batch of 200 g. cod intestine yielding 2.5 g. dry ammonium sulphate precipitate with a total activity of 1,100 U. (5.5 U./g. wet weight) was subjected to the purification procedure described by Pernow (1953). After extraction with 70% methanol the solution was passed through a column of 12 g. aluminium oxide. Some eluates were pooled, concentrated *in vacuo*, and made up to a concentration of 70% methanol and again run through an aluminium oxide column and eluted with decreasing concentrations of methanol. The results obtained are shown in Table I.

Biological Activity.—The effects of a partially purified extract from cod intestine, consisting of

TABLE I

BIOLOGICAL ACTIVITY IN ELUATES FROM A PARTIALLY PURIFIED EXTRACT OF COD INTESTINE CONTAINING 480 U. "P"

The assays were performed on guinea-pig gut in the presence of atropine and lergigan.

Methanol Concentration of Elution Fluid	Volume (ml.)	Activity (U.) after Passing Through Column
Concentrated eluates in 70% methanol	25	< 10
70%	15	< 10
60%	19	10
50%	27	378
40%	14	28
20%	15	< 10
20%	16	< 10
aq. dest.	15	< 10
		Total 416 U.

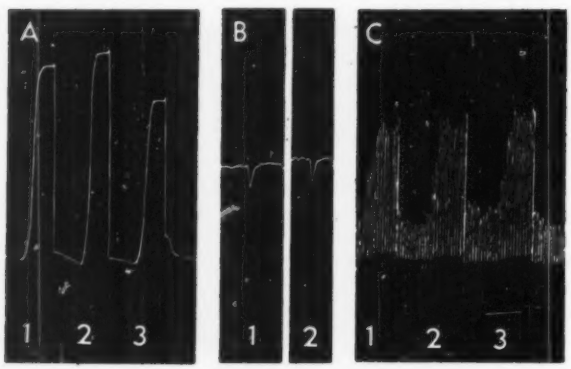

FIG. 1.—A. Isolated guinea-pig ileum. 3 ml. bath. 38° C. Atropine and lergigan 1 : 2.5 mill. in Tyrode solution. 1, 0.2 U. "P" standard. 2, 0.008 ml. purified cod intestine extract "C." 3, 0.007 ml. same extract. B. Blood pressure, rabbit, atropine. 1, 0.06 ml. extract "C." 2, 2 U. "P." C. Isolated rabbit jejunum. 15 ml. bath. Atropine 1 : 2.5 mill. in Tyrode solution. 38°. 1, 0.03 ml. extract "C." 2, 1 U. "P." 3, 0.04 ml. extract "C."

eluates obtained with 50% methanol and lower concentrations, concentrated *in vacuo*, were tested on various biological preparations and compared with a Substance P standard. The extract contained 9.3 U. "P"/mg. dry weight. The results are presented in Table II and Fig. 1. In all instances good agreement between the general type of effect of the cod intestinal extract and Substance P standard was obtained. No difference was noted

TABLE II

BIOLOGICAL ACTIVITY OF PURIFIED COD INTESTINE EXTRACT

Test Preparation	Effect	Activity (Units "P"/ml.)
Guinea-pig ileum (atropine-lergigan) ..	Contraction	24–27
Rabbit duodenum (atropine)	,,	25
Rabbit blood pressure (atropine) ..	Slight fall	20
Cat blood pressure (atropine, lergigan)	,, ,,	20–25

between the biological effects of the extract before and after atropine and lergigan, showing that for practical purposes it was free from choline and choline esters or histamine. Since the effect was completely abolished by treatment with trypsin, no other active substance of non-polypeptide nature (such as 5-hydroxytryptamine) seemed to be present.

Inactivation by Trypsin.—Incubation of purified cod intestinal extracts with pure trypsin 1 : 3,000 caused half-inactivation in about 10 min. at *p*H 5.5 at 38° C. in the same way as for Substance P from mammalian intestine. After treatment with trypsin the inactivation was proportional on all test preparations, proving the protein (polypeptide) nature of the active substance. Fig. 2 shows the activity of the extract on rabbit duodenum and guinea-pig ileum after treatment with trypsin.

A quantitative comparison of the cod intestinal extract and Substance P standard on the various test preparations showed that good agreement was obtained on guinea-pig ileum, rabbit duodenum, and on cat and rabbit blood pressure. An equally good agreement was also noted between the effects of Substance P standard and two other cod intestinal extracts on the test preparations used. All of the extracts were inactivated by trypsin.

Dogfish Intestine

From 13 dogfish weighing about 25 kg., 270 g. intestine were obtained, yielding 3.55 g. dry ammonium sulphate precipitate. After this was dissolved in water and methanol added to 70%, the clear supernatant after centrifugation contained 1,300 U. (4.8 U./g.).

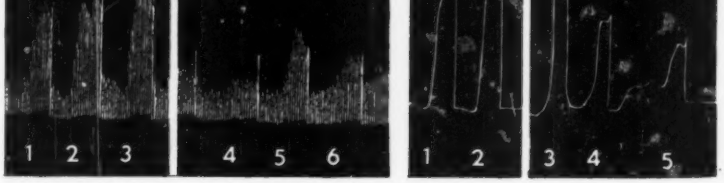

FIG. 2.—A and B. Isolated rabbit jejunum. 15 ml. bath. Atropine 1 : 2.5 mill. in Tyrode solution. 38° C. 1, 0.05 ml. purified cod intestine extract "B." 2, 1 U. "P." 3, 0.08 ml. extract "B." 4 (½ hr. later), 0.067 ml. extract "B" incubated with trypsin. 5, 0.2 U. "P." 6, 0.1 U. "P." C and D. Isolated guinea-pig ileum. 3 ml. bath. Atropine and lergigan 1 : 2.5 mill. in Tyrode solution. 1, 0.3 U. "P." 2, 0.02 ml. extract "B." 3 (45 min. later), 0.1 U. "P." 4, 0.05 U. "P." 5, 0.02 ml. extract "B" incubated with trypsin.

The methanol extract was run through an aluminium column as described above and eluted with decreasing concentrations of methanol. Most of the activity was found in the 20% methanol and aqueous fractions.

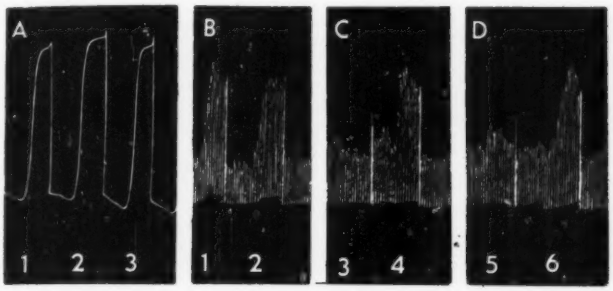

FIG. 3.—A. Isolated guinea-pig ileum. 3 ml. bath. Atropine and lergigan. 1, 0.1 ml. purified cod brain extract "D." 2, 0.2 U. "P." 3, 0.05 ml. purified dogfish intestine extract "E." B.–D. Isolated rabbit jejunum. 15 ml. bath. Atropine. 1, 0.4 ml. extract "D." 2, 1 U. "P." 3, 0.25 ml. extract "E" incubated with trypsin. 4, Same as 3 without incubation. 5, 0.18 ml. extract "D" incubated with trypsin. 6, 0.18 ml. "D."

After removal of the methanol the action of these fractions was tested on various biological preparations and good agreement was found on comparison with Substance P standard (Fig. 3). Treatment with trypsin abolished the effect completely.

Cod Brain

A small amount of cod brain, 15.3 g. from 13 cods weighing 15 kg., was extracted and precipitated with ammonium sulphate as above. An aqueous solution of the precipitate was used for direct assay on the same preparations as used for the extracts of intestine and showed the same type of actions. The activity was 6 U./g. fresh brain on all test preparations. After incubation with trypsin no effect was obtained on the test preparations.

Fig. 3 illustrates the effect of the cod brain extract, purified by adsorption on aluminium oxide and eluted with methanol in decreasing concentrations, on guinea-pig ileum and rabbit duodenum.

DISCUSSION

A factor has been found in ammonium sulphate precipitates of fish intestine and brain which stimulated the isolated intestine of the guinea-pig and rabbit and lowered the rabbit's blood pressure, thereby suggesting that this activity was due to Substance P. This assumption was strongly supported by the facts that, after purification, the factor had the same actions as Substance P qualitatively and quantitatively, even in the presence of atropine and antihistamine drugs, and was inactivated by trypsin in the same way as was Substance P. Evidence has recently been produced by Eliasson, Lie, and Pernow (1956) to show that Substance P in mammalian intestine is identical with that in brain. The present data have not revealed any differences between the Substance P activity from intestine and brain in fish.

It seems therefore that Substance P is not restricted to mammals but also occurs in considerable quantities in the corresponding organs of fish.

SUMMARY

1. A factor having, qualitatively and quantitatively, the same properties as Substance P has been shown to occur in the intestine of the cod and the dogfish and in cod brain.

2. The activity of the ammonium sulphate precipitate corresponded to 4.8–5.5 Units "P"/g. intestine and to 6 U. "P"/g. brain.

3. The active principle was purified by adsorption on aluminium oxide and elution with methanol in decreasing concentrations.

4. The active principle was inactivated by incubation with trypsin in a similar way to Substance P from mammalian intestine.

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THE EFFECT OF CORTISONE ALONE AND IN COMBINATION WITH ISONIAZID ON EXPERIMENTAL MURINE LEPROSY IN MICE

BY

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(RECEIVED MAY 28, 1956)

There has been much work on the effects of cortisone on tuberculosis but no publications on its effects on experimental leprosy. Recently, Naguib and Robson (1955) reported that cortisone exacerbates corneal tuberculosis in mice and overcomes the beneficial effects of isoniazid. In rabbits, Greenburgh, Robson, and Willcox (1953) had previously found that cortisone decreases the severity of corneal tuberculosis. The purpose of the present investigation was to determine the effect of the drug on the development of murine leprosy induced by the intracorneal inoculation of the Mycobacterium in mice.

METHODS

Details of the method used for corneal inoculation with *Myco. lepraemurium* have been described previously (Naguib, Rees, and Robson, 1956). The leprosy material used in the present investigation was obtained from a mouse which had been infected intracorneally from a rat leproma (Douglas strain) and had developed generalized leprosy. The mouse was killed and a suspension in 0.05% Tween 80 saline was prepared from the ground liver and spleen. The inoculum, which was about 0.01 ml., contained approximately 2.3×10^{11} lepra bacilli. Albino mice of the "C" strain of 25 g. average weight were used and were divided into four groups: (1) 10 controls, (2) 10 treated with isoniazid, (3) 16 treated with cortisone, and (4) 16 treated with cortisone and isoniazid. Treatment was started on the day of inoculation. The isoniazid therapy was continued until the end of the experiment (40 weeks). All mice on cortisone (groups 3 and 4) received the drug for 55 days. At the end of this period the surviving mice of groups 3 and 4 were divided into equal subgroups: in one the cortisone was discontinued, and in the other the cortisone was administered until all animals had died.

Cortisone acetate suspended in saline was injected subcutaneously in daily doses of 0.5 mg./mouse for 6 days/week; isoniazid was mixed in M.R.C. diet 41 and given in a dose of 0.3 mg./mouse/day in 5 g. of diet. To prevent secondary infection after corneal inoculation aureomycin was given in the drinking-water in a dose of 100 mg./kg. body weight (on the assumption that each mouse drinks 4 ml. daily) and was continued throughout the experiment.

The lesions which developed on the cornea were examined daily and the size estimated in arbitrary units. Values of 0.6 and under do not represent true lesions, which are represented by the higher values given to areas of denser opacity in the cornea.

RESULTS

The intracorneal inoculation of leprosy material produced an initial reaction in all the mice in the form of an opacity which faded gradually until the true lesion became superimposed upon it.

Control Animals.—Corneal lesions developed as small foci in the centre of the opaque areas in all the control mice after an incubation period of some 17 days. They then increased in size and density over the cornea and spread to the sclera, which became thickened and swollen. The progress of these lesions is shown in Fig. 1.

All the mice of this group survived for periods varying between 23 and 31 weeks. At post-mortem examination they all had severe generalized leprosy involving many of the internal organs, particularly the liver and spleen. In one of the animals leprosy bacilli were found in the spinal cord.

Isoniazid.—The mice treated with isoniazid developed lesions after the same latent period as the controls. These lesions progressed very slowly and remained small, except in one animal, in which two-thirds of the cornea was involved at one stage of the infection. Prolonged treatment resulted in a decrease in both the size and density of the lesions, which in two mice almost disappeared, leaving an opacity similar to the initial reaction. One of these mice was killed at 23 weeks for histological examination and another died at 31 weeks. The other eight were alive and well at 41 weeks, when they were killed for post-mortem

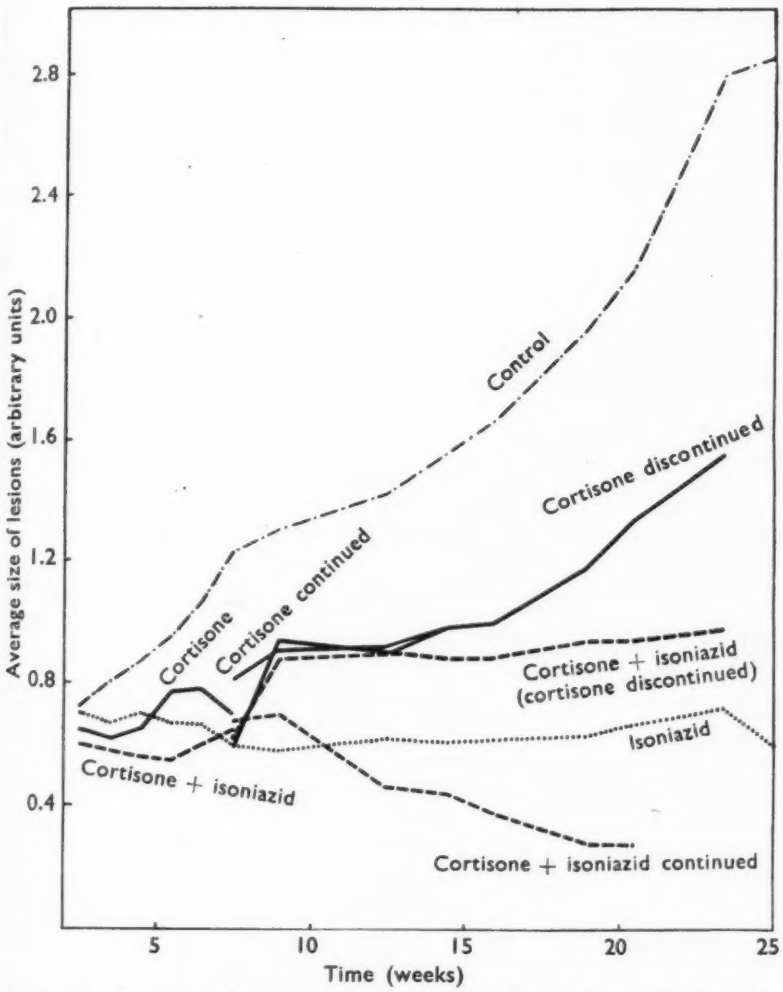

FIG. 1.—Showing the development of corneal lesions in the various groups of animals. — · — · — Control group. . . . . Isoniazid group. ———— Animals on cortisone; 55 days after inoculation the surviving animals were divided into two subgroups, in one of which the cortisone was discontinued and in the other continued. - - - - - - - Animals on cortisone and isoniazid. The isoniazid was given until the end of the experiment. At 55 days the surviving animals were divided into two subgroups, in one of which the cortisone was discontinued and in the other continued.

examination. In six of them no evidence of infection, either macroscopic or microscopic, was found in the various internal organs. In the other two a few bacilli were found on microscopic examination of the liver and spleen, but there were no macroscopic lesions. The isoniazid treatment had thus produced a striking effect on the course of the infection, both in the cornea and in the internal organs.

Cortisone.—The latent period preceding the appearance of lesions was considerably increased, and for 7 weeks no typical lesions were seen. One of the mice died 17 days after inoculation and showed no changes in the cornea. In all the others, blebs appeared in the cornea in the period preceding the development of typical lesions. The bleb usually appeared as a transparent spot in the centre of an opaque area (the site of inoculation), but sometimes elsewhere in the cornea, and in a number of mice more than one bleb developed. The bleb grew and became filled with fluid, resembling a blister. Some of the blebs disappeared and reappeared after various periods. They never became herniated, and never spread to destroy the cornea and the eye as has been seen in corneal tuberculosis treated with cortisone (Naguib and Robson, 1955).

Fifty-five days after inoculation 14 animals of this group were still alive and in seven the cortisone was continued. Two of these died 9 weeks after inoculation without developing corneal lesions, and the other 5 developed corneal lesions at periods after inoculation varying from 87 to 144 days. In the animals in which the cortisone was discontinued, true lesions appeared about 10 days after cessation of treatment, except in one animal where the latent period was much longer (110 days). The average size of the lesions in the various groups is shown in Fig. 1.

Five of the animals on continued cortisone survived for periods varying between 15 and 23 weeks. At post-mortem examination comparatively few macroscopic lesions were found and these were small and scattered ; sections or smears of the liver and spleen of 3 of the animals which died 16–21 weeks after inoculation showed a large number of lepra cells filled with leprosy bacilli. The effect in these animals, therefore, is similar to that described by Lurie and Zappasodi (1955) in rabbits infected by inhalation of *M. tuberculosis* and treated with cortisone.

The mice in which cortisone was discontinued survived for periods varying between 23 and 36 weeks, and in all but one of these the macroscopic

lesions were either small and not numerous, or totally absent. In 5 out of 7 animals, however, section or smears showed many lepra cells with very large numbers of bacilli. In the other 2 mice, the number of bacilli found in the liver and spleen was appreciably smaller.

Cortisone and Isoniazid.—No true corneal lesions appeared during the first 7 weeks following inoculation, and bleb formation was seen as in the mice on cortisone alone. Six of these animals died before 55 days. In 4 of the animals in which cortisone was continued, no corneal lesions developed and in one a lesion appeared 87 days after inoculation. On the other hand, in 4 of the animals in which cortisone was discontinued, true corneal lesions appeared at periods varying from 62 to 101 days after inoculation; in the fifth the cornea remained clear until the end of the experiment.

The animals in which cortisone was continued survived for periods varying between 19 and 25 weeks. On post-mortem examination no macroscopic lesions were present in the internal organs in 4 out of 5 of the mice, and in the fifth a single minute macroscopic lesion was found in the liver. Smears or sections of the liver and spleen of these animals showed either a few bacilli (in 3) or none at all (in the other 2).

The animals in which cortisone was discontinued survived for periods varying between 23 and 40 weeks. In 2 of these, post-mortem examination revealed extensive macroscopic involvement of the liver and spleen, but in the other 3 no macroscopic lesions were found. Smears from the organs of the animals with gross macroscopic lesions showed huge numbers of lepra cells with many bacilli, but in the other 3 animals few or no bacilli were recovered.

Discussion

Inoculation of *M. lepraemurium* into the cornea of mice leads to progressive involvement of the eye and systemic spread of the disease, which ultimately kills the animals. Administration of isoniazid has a striking inhibitory effect on the course of both the ocular and systemic infections.

Cortisone modifies the spread of murine leprosy; but the effects are rather complex and the results are summarized in Table I. Cortisone delayed the appearance of macroscopic lesions, both locally and systemically. When its administration was stopped at 55 days, lesions quickly made their appearance in the cornea, as was evident from the sudden increase in the density of the corneal opacities; this increase was not seen in animals on continued cortisone therapy. The inhibitory effect on the spread of the corneal lesions is similar to that obtained with corneal tuberculosis in the rabbit (Greenburgh *et al.*, 1953).

The systemic lesions in the liver and spleen were much smaller than in the untreated animals, but sections or smears from these were swarming with bacilli.

The combination of cortisone with isoniazid produced effects similar to those produced by isoniazid alone; the adrenal steroid did not overcome the beneficial effect of isoniazid. On the other hand, and rather unexpectedly, when the cortisone administration was stopped at 55 days, two of the animals on continued isoniazid (which survived respectively for 23 and 41 weeks) did develop gross systemic leprosy, which was never seen in animals on isoniazid alone. This suggests that occasionally cortisone can reverse the effect of isoniazid, an action similar to that consistently observed in tuberculous infection arising in the cornea (Naguib and Robson, 1955).

TABLE I

SHOWING THE COURSE OF THE INFECTION IN THE VARIOUS GROUPS OF ANIMALS

(For further information see text)

Group	No. of Mice	Mean Survival Time (Weeks)	Cornea	Systemic Disease
Controls	9*	27	Progressive disease	Gross macroscopic lesions
Isoniazid	8†	40	Small lesions	Nil or very slight
Cortisone for whole period	7‡	16	Small delayed lesions ‖	Small macroscopic lesions. Masses of bacilli
Cortisone discontinued at 55 days	7	26	Lesions showing after‖ discontinuation of cortisone	Idem
Cortisone + isoniazid for whole period	5§	22	Nil or slight‖	Slight or nil
Cortisone discontinued at 55 days	5	36	Lesions at 7–46 days‖ after discontinuation of cortisone in 4 animals	Gross macroscopic lesions in 2 mice. Slight or nil in 3

* 1 killed for histology. † 1 killed for histology; 1 died at 3 weeks. ‡ 2 excluded which died at 2–3 weeks. § 6 excluded which died at 3–7 weeks.
‖ Apart from blebs in early stage.

The development of blebs in the cornea in the early stages of cortisone therapy, both with and without isoniazid, is puzzling. The similar phenomenon observed in tuberculosis of the cornea in animals receiving cortisone was accompanied by multiplication of the bacilli (Naguib and Robson, 1955; Robson and Didcock, 1956) so that the area of bleb formation contained a mass of bacilli. It seemed possible that this led to the production of some toxic substance responsible for the bleb formation and subsequent perforation of the cornea. In the leprosy infection, however, bleb formation occurred in the absence of any true macroscopic lesion and never was the prelude to perforation and destruction of the eye. The mechanism of bleb formation in the cornea under these circumstances requires further investigation.

One last point deserves mention. It has been found that in some cases of human leprosy, particularly in leprous eye inflammation, cortisone may be of value to supplement other drug treatment (Lowe, 1952). This is in keeping with the results of the present experiments.

Summary

1. Mice were inoculated intracorneally with *M. lepraemurium* and treated with cortisone, or with a combination of cortisone and isoniazid.

2. Cortisone decreased the development of both macroscopic corneal and systemic lesions; nevertheless, the liver and spleen in most animals contained very large numbers of lepra cells filled with bacilli. Cortisone did not appreciably modify the beneficial action of isoniazid, except in two mice in which gross systemic lesions occurred; this was not seen in mice on isoniazid alone.

The expenses of this investigation were in part defrayed by a grant from the Medical Research Council (to J.M.R.). Technical assistance was provided by a grant from Guy's Hospital Endowment Fund.

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TRYPANOCIDAL ACTION OF PHENANTHRIDINE COMPOUNDS: EFFECT OF CHANGING THE QUATERNARY GROUPS OF KNOWN TRYPANOCIDES

BY

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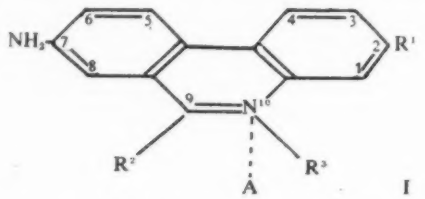
Woolfe (1952) showed that the trypanocidal action of dimidium bromide (2:7-diamino-10-methyl-9-phenylphenanthridinium bromide; I, R^1=—NH_2, R^2=phenyl, R^3=—CH_3, A=—Br) is profoundly affected by changing the quaternary group. The corresponding ethyl quaternated compound (I, R^1=—NH_2, R^2=phenyl, R^3=—C_2H_5, A=—Br), to which the name ethidium bromide has been given, was far more effective as a trypanocide



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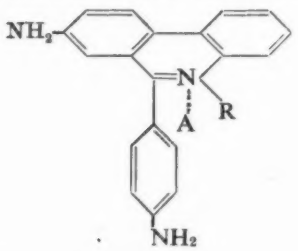
in mice against all the African species of trypanosomes used. Field trials in Africa have shown that ethidium is more active than dimidium against *Trypanosoma congolense* infections in cattle (Wilde and Robson, 1953; Wilson and Fairclough, 1953; Ford *et al.*, 1953a; Karib *et al.*, 1954; Milne and Robson, 1955) and against *T. vivax* infections in cattle (Ford *et al.*, 1953b; Unsworth, 1954a, b). In numerous experiments ethidium has also been shown to be less toxic than dimidium to cattle (Burdin and Plowright, 1952; Burdin, 1953; Ford *et al.*, 1954; Wilson, 1954). It was considered important, therefore, to try the effect of changing the quaternating group of other phenanthridinium compounds which, in the methyl-quaternated form, had been found to possess trypanocidal activity.

METHODS

The techniques used were the same as those described previously (Woolfe, 1952). Toxicities were determined by subcutaneous injection in mice. Trypanocidal

TABLE I

THE TRYPANOCIDAL ACTIVITY OF PHENIDIUM DERIVATIVES



II

Compound	Structure		LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide=1)			
	R	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
Phenidium chloride	methyl	Cl	60	0·3	Delayed death at 40 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 40 mg./kg.
RD2057	ethyl	Cl	100	0·5	,, ,,	0·1	,, ,,
RD2081	*n*-propyl	Cl	100	0·25	,, ,,	0·2	,, ,,
RD2075	allyl	Cl	70	0·6	,, ,,	0·15	,, ,,

activities recorded were determined by giving a single subcutaneous dose to mice which, after inoculation with diluted blood of infected mice, showed a moderate degree of parasitaemia. The Median Curative Dose (CD50) was estimated as the dose necessary to remove parasites from the peripheral blood of 50% of mice for a period of not less than one month.

RESULTS

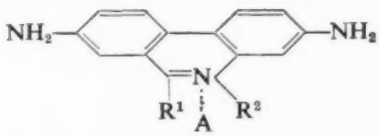
In the Tables which follow, the activity of each new compound (where activity was found) has been compared on the basis of the CD50 with that of dimidium bromide, the activity of which was taken as unity. For instance, if in one test the CD50 of dimidium bromide was 0.7 mg./kg., and that of the new compound 0.35 mg./kg., the activity would be given as 2.0. Figures in the Tables represent the means of all the tests carried out, although when a particular compound showed complete inactivity against any trypanosome species the test was not repeated.

Derivatives of Phenidium

Phenidium chloride (7-amino-9-*p*-amino phenyl-10-methylphenanthridium chloride; I, R^1=—H, R^2=*p*-aminophenyl, R^3=—CH_3, A=—Cl) was used against *T. congolense* in Africa until it was replaced by the more active compound, dimidium. However, phenidium is less toxic than dimidium, and the view has been expressed that phenidium

TABLE II

THE TRYPANOCIDAL ACTIVITY OF 2:7-DIAMINO PHENANTHRIDINIUM COMPOUNDS



Group	Compound	Structure			LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide = 1)			
		R^1	R^2	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
a	Dimidium bromide	phenyl	methyl	Br	85	1	1	1	1
	Ethidium bromide	,,	ethyl	Br	(110)	(10)	(20)	(11)	(50)
	RD1427	,,	*n*-propyl	Br	(100)	(6)	(35)	(12)	(40)
	RD1446	,,	allyl	Br	(115)	(6)	(20)	(18)	(30)
b	150C47	*p*-amino phenyl	methyl	Cl	>200	0·4	<0·1	<0·1	<0·1
	RD1698	,,	ethyl	Cl	140	1·5	5	2 5	3
	RD1629	,,	*n*-propyl	Cl	140	2·2	9	6	2·5
	RD1595	,,	allyl	Cl	120	3·2	2	6	3
c	676C46	*p*-nitro phenyl	methyl	Cl	120	0·5	0·4	0·6	0·4
	RD1662	,,	ethyl	Cl	140	0·6	5	3	3
	RD1601	,,	*n*-propyl	Cl	280	0·6	3	6	4
	RD1542	,,	allyl	Cl	*ca.* 400	2·5	6	8	8
d	660C47	benzyl	methyl	Br	80	0·85	0·12	0·3	0·2
	RD2607	,,	ethyl	Br	90	3·5	1·2	4	8
e	621C47	2-thienyl	methyl	Br	[12 i.v.]	[1·3]		[<1]	
	RD2482	,,	ethyl	Br	100	14	5	12	7
f	492C46	methyl	methyl	SO_4CH_3	70	0·1	Inactive at 32 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.
	RD2234	,,	ethyl	Br	125	0·125	Delayed death at 32 mg./kg.	0·03	,,
	RD2319	,,	allyl	I		0·03	Inactive at 32 mg./kg.	Delayed death at 32 mg./kg.	,,

Figures in parentheses (x) are taken from Woolfe (1952). Figures in brackets [x] are taken from Brownlee *et al.* (1950).

may have been discarded prematurely (Burdin, Plowright, and Purchase, 1952). As I found -ethyl, -propyl, and -allyl to be the most useful replacement quaternating groups in dimidium, with activity reduced by longer chains, only these groups were tried for phenidium.

The results obtained are shown in Table I. It will be seen that phenidium itself was less than one-third as active as dimidium against *T. congolense*, and almost inactive against *T. brucei*, *T. rhodesiense* and *T. gambiense*. Changing the quaternating group to -ethyl (RD 2057) or -allyl (RD 2075) increased activity against *T. congolense* about twofold, but the propyl derivative (RD 2081) had substantially the same activity as had phenidium. All three derivatives had increased activity against *T. rhodesiense*, though they were still inferior to dimidium. Activity against *T. brucei* and *T. gambiense* was little changed.

Derivatives of Known 2:7-*Diaminophenanthridinium Compounds*

Brownlee *et al.* (1950) published results of tests on a number of phenanthridinium compounds, some of which they found to be more active than dimidium. These latter compounds were recently tried in the field in Africa (Goodwin and Chandler, 1952; Goodwin and Unsworth, 1952; Neal and Karib, 1954). I had, unwittingly, duplicated some of the original work, and had agreed, in the main, with the published results, though not finding 150C47 and 676C46 (see Table II) to be more active than dimidium. However, I thought that it would be useful to try the effect of changing the quaternary group on some of these compounds, and several derivatives were made. I tried some of the less active compounds as well as those said to be more active than dimidium; Table II summarizes the results obtained. For comparison, the dimidium derivatives are included in the same Table.

Group (*a*), 9-*Phenyl Derivatives.*—As was shown previously, the trypanocidal activity of the parent compound, dimidium bromide, is increased many-fold in the ethyl, propyl and allyl quaternated compounds.

Group (*b*), 9-p-*Aminophenyl Derivatives.*—Brownlee *et al.* (1950) found the methyl quaternated compound to possess trypanocidal activity greater than that of dimidium; we found it less active than dimidium. However, the ethyl, propyl, and allyl quaternated compounds were several times more active than dimidium against *T. congolense*, *T. brucei*, *T. rhodesiense*, and *T. gambiense*.

Group (*c*), 9-p-*Nitrophenyl Derivatives.*—Again Brownlee *et al.* stated the methyl quaternated compound to be more potent than dimidium, and again we differed. Against *T. congolense* the ethyl and propyl compounds were less active than dimidium, but the allyl derivative was more active. Against the other trypanosome species all three new compounds were more active than dimidium or the parent methyl compound.

Group (*d*), 9-*Benzyl Derivatives.*—Only the ethyl derivative was prepared. This appeared to be considerably more active than the methyl derivative.

Group (*e*), 9-*Thienyl Derivatives.*—The ethyl quaternated compound had activity of a very high order against *T. congolense*, and it was more active than dimidium against all the species of trypanosomes used.

Group (*f*), 9-*Methyl Derivatives.*—The parent methyl compound had negligible activity. Slight but definite activity against *T. congolense* and *T. rhodesiense* resulted after ethyl quaternation; allyl quaternation was less effective.

It is obvious from the results given, that the effect of the quaternary group on the trypanocidal activity of phenanthridium compounds is of major importance. The enhancement of activity produced by replacing a methyl group by, say, ethyl is greatest when the original compound itself has high activity; thus in the 9-phenyl series ethidium is very much more active than dimidium, but in the 9-methyl series the ethyl compound has only slight activity. We are still investigating the reason for this increase in activity.

Summary

1. The effect on trypanocidal activity of replacing the methyl quaternating group of known phenanthridinium compounds by ethyl, propyl, or allyl has been tried.

2. Trypanocidal activity was always enhanced by such changes, and the increase in activity was greatest with compounds originally of high activity.

I am grateful to Dr. T. I. Watkins, who supplied all the compounds here reported; the chemistry of this work will be published elsewhere. My thanks are due, too, to Dr. M. R. Gurd, who supplied the toxicity figures for the various compounds.

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Brit. J. Pharmacol. (1956), **11**, 334.

TRYPANOCIDAL ACTION OF PHENANTHRIDINE COMPOUNDS: FURTHER 2:7-DIAMINO PHENANTHRIDINIUM COMPOUNDS

BY

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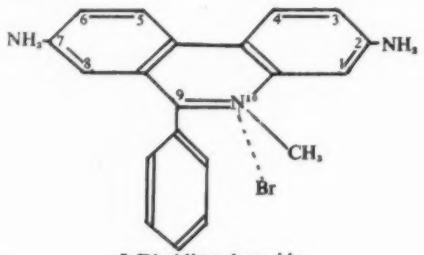
(RECEIVED JUNE 14, 1956)

Woolfe (1952) showed that the trypanocidal activity of dimidium bromide (2:7-diamino-10-methyl-9-phenyl-phenanthridinium bromide; I) was influenced by changing the quaternary group, and later (Woolfe, 1956) that the activity of other



I Dimidium bromide

trypanocidal phenanthridinium compounds was also modified by such changes. Results obtained with a number of other 2:7-diamino phenanthridines are now reported.

METHODS

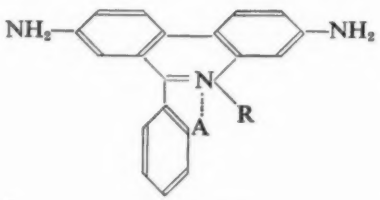
The techniques described earlier (Woolfe, 1952) were used, and drugs were given in single doses subcutaneously to mice. The Median Curative Dose (CD50) was estimated as the dose necessary to remove parasites from the peripheral blood of 50% of infected mice for a period of not less than one month. Active drugs were compared with dimidium (activity taken as 1) on the basis of the CD50.

RESULTS

2 : 7-*Diamino-9-phenyl-phenanthridinium Compounds* (Table I).—I previously (Woolfe, 1952) discussed the effect of *n*-alkyl and of allyl-quaternating groups. I have now tried a number of quaternating groups containing hetero-atoms, and in the

TABLE I

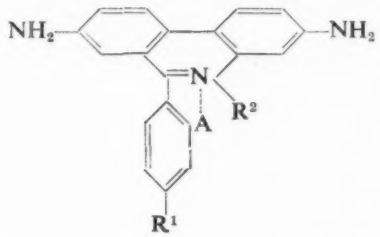
THE TRYPANOCIDAL ACTIVITY OF 2:7-DIAMINO-9-PHENYL-PHENANTHRIDINIUM COMPOUNDS



Compound	Structure		LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide = 1)			
	R	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
RD2443	$CH_2.CH_2OH$	SO_3CH_3		2·0	0·7	2·2	1·6
RD2490	$CH_2CH_2O.CH_3$	SO_3CH_3	55	0·7	1·0	2·2	0·8
RD2505	$CH_2CH_2O.C_2H_5$	SO_3CH_3	80	1	0·3	2·5	0·8
RD2141	$CH_2CH_2CH_2.O.C_2H_5$	SO_3CH_3	400	6	6	8	8
RD1630	$CH_2CH_2CH_2.N(C_2H_5)_2$	SO_3CH_3	100	1·2	1	*ca.* 0·5	1
RD1667	$CH_2CH_2CH_2.N(C_2H_5)_2$ with CH_3	I	16	< 0·2	Inactive at 8 mg./kg.	0·3	Inactive at 8 mg./kg.

TABLE II

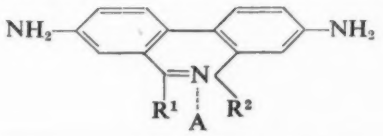
THE TRYPANOCIDAL ACTIVITY OF 2: 7-DIAMINO-9-(*p*-SUBSTITUTED PHENYL) PHENANTHRIDINIUM COMPOUNDS



Compound	Structure			LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide = 1)			
	R^1	R^2	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
RD1847	Cl	CH_3	Cl	80	0·125	Inactive at 16 mg./kg.	0·2	Inactive at 16 mg./kg.
RD1870	Cl	$CH_2CH=CH_2$	Cl	100	0·3	1·2	2	2
RD2177	CH_3	CH_3	Br	14	*ca.* 0·02	Delayed death at 8 mg./kg.	0·1	Delayed death at 8 mg./kg.
RD2201	CH_3	C_2H_5	Br	80	*ca.* 0·05	0·3	0·4	0·3
RD1911	$CH(CH_3)_2$	$CH_2CH=CH_2$	Cl	40	Inactive at 16 mg./kg.	Inactive at 16 mg./kg.	Inactive at 16 mg./kg.	Inactive at 16 mg./kg.
RD1535	OCH_3	CH_3	Br	35	*ca.* 0·1	*ca.* 0·1	*ca.* 0·2	Delayed death at 16 mg./kg.
RD2022	$SO_2C_2H_5$	CH_3	SO_3CH_3		0·6	*ca.* 1	*ca.* 1·3	*ca.* 1
RD2056	$SO_2C_2H_5$	C_2H_5	Cl	100	Temporary clearing at 20 mg./kg.	Inactive at 32 mg./kg.	Delayed death at 20 mg./kg.	Inactive at 32 mg./kg.
RD2035	$SO_2C_2H_5$	$CH_2.CH=CH_2$	SO_3CH_3		Inactive at 20 mg./kg.	Delayed death at 32 mg./kg.	,,	Delayed death at 32 mg./kg.

TABLE III

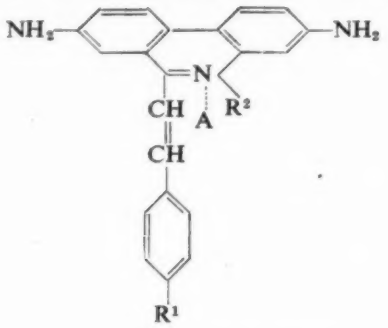
THE TRYPANOCIDAL ACTIVITY OF 2: 7-DIAMINO-9-ALKYL-PHENANTHRIDINE COMPOUNDS

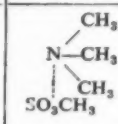
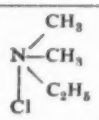


Compound	Structure			LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide = 1)			
	R^1	R^2	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
RD2610	C_2H_5	CH_3	Br	160	0·1	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.
RD2611	C_2H_5	C_2H_5	Br	250	0·22	,,	0·1	,,
RD1098	$C_4H_9(n)$	—	—	80	Inactive at 30 mg./kg.	Inactive at 30 mg./kg.	Inactive at 30 mg./kg.	Inactive at 30 mg./kg.
RD1120	$C_4H_9(n)$	CH_3	Br	70	Temporary clearing at 20 mg./kg.	Inactive at 20 mg./kg.	Inactive at 20 mg./kg.	Inactive at 20 mg./kg.
RD2652	C_9H_{19}	CH_3	Cl		Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.
RD2729	C_9H_{19}	C_2H_5	Cl		Temporary clearing at 32 mg./kg.	,,	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.

TABLE IV

THE TRYPANOCIDAL ACTIVITY OF 2:7-DIAMINO-9-STYRYL PHENANTHRIDINIUM COMPOUNDS



Compound	Structure			LD50 mg./kg. (s.c.)	Trypanocidal Activity (Dimidium Bromide = 1)			
	R^1	R^2	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
RD2345	H	CH_3	SO_3CH_3	*ca.* 200	0·02	0·2	0·03	0·1
RD2585	H	C_2H_5	SO_3CH_3		0·03	0·2	0·07	0·2
RD2337	NH_2	CH_3	Br	*ca.* 100	0·03	1·2	8	6
RD2480	NH_2	C_2H_5	Br	*ca.* 250	0·15	20	12	14
RD2240	$N(CH_3)_2$	CH_3	SO_3CH_3	250	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	0·06	0·1
RD2384	$N(CH_3)_2$	C_2H_5	Cl		,,	0·1	0·3	0·25
RD2289		CH_3	SO_3CH_3	60	Delayed death at 16 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	0·1
RD2415		C_2H_5	Cl	50	0·025	0·25	0·25	0·1

alkoxyalkyl series the ethoxypropyl compound, RD 2141, was particularly interesting, having low acute toxicity for mice and high trypanocidal activity. The diethylaminopropyl compound, RD 1630, had activity of the same order as that of dimidium, but when the nitrogen was quaternated, in RD 1667, toxicity increased and activity decreased.

2:7-*Diamino*-9-(*p-substituted Phenyl*) *Phenanthridinium Compounds* (Table II).—Two groups of this type, namely the *p*-amino phenyl and the *p*-nitro phenyl compounds, were previously discussed (Woolfe, 1956). Apart from these two *p*-substituents every other substituent tried has reduced activity below that of the parent dimidium.

I found that modifying the methyl quaternary group increases activity in the *p*-chlorophenyl and *p*-methylphenyl compounds; but in the *p*-ethyl sulphonyl phenyl compounds, methyl quaternation (RD 2022) produced greater activity than either ethyl or allyl quaternation.

2:7-*Diamino*-9-*alkyl Phenanthridinium Compounds* (Table III).—I have already shown (Woolfe, 1956) that the 9-methyl compounds had but slight activity. I have now tried 9-ethyl, 9-*n*-butyl and 9-nonyl compounds. None of these had more than a trace of trypanocidal activity, and ethyl quaternation in place of methyl, though it did increase activity to some extent, could not produce useful activity. The one non-quaternized compound reported here, RD 1098, was completely devoid of trypanocidal activity.

2:7-*Diamino*-9-*styryl Phenanthridinium Compounds* (Table IV).—It was thought possible that the degree of conjugation of the phenanthridinium compounds might bear some relationship to trypanocidal activity, in which case styryl derivatives should be more active than the corresponding phenyl

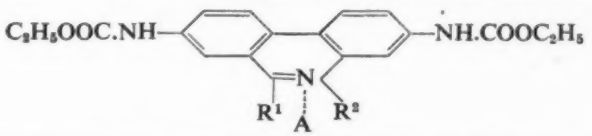
compounds. Accordingly a number of 9-styryl-phenanthridinium compounds was prepared. Each was less active than the corresponding 9-phenyl compound against *T. congolense*, and ethyl quaternation in place of methyl increased activity only to a minor extent. However, the 9-*p*-amino styryl compounds had surprisingly high activity against other trypanosome species.

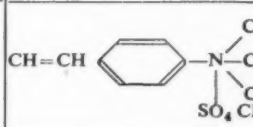
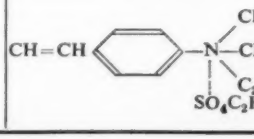
2 : 7-*Dicarbethoxyamino Phenanthridinium Compounds* (Table V).—It has been reported previously that interference with the primary amino groups in the 2: 7-positions greatly reduces trypanocidal activity against African species of trypanosomes, though such compounds may possess activity against the South American *T. cruzi* (Goodwin *et al.*, 1950). I have prepared and tested a number of such compounds. None here reported showed more than a trace of activity against *T. congolense*, *T. brucei*, *T. rhodesiense*, or *T. gambiense*, though several of the parent primary amines were highly active.

It is obvious, from results presented both here and in my previous papers on this subject, that the quaternating group in phenanthridinium compounds may play a very large part in determining the activity of the compound. In every case, except that of the 9-ethyl sulphonyl phenyl compounds, the methyl derivative was less active as a trypanocide than the ethyl, *n*-propyl or allyl-derivatives. The reason for the reversal of this effect in the 9-ethyl sulphonyl phenyl series is not yet apparent. The high activity and low toxicity of the ethoxypropyl

TABLE V

THE TRYPANOCIDAL ACTIVITY OF 2: 7-DICARBETHOXYAMINO PHENANTHRIDINIUM COMPOUNDS



Compound	Structure			LD50 mg./kg. (s.c.)	Trypanocidal Activity			
	R^1	R^2	A		*congolense*	*brucei*	*rhodesiense*	*gambiense*
RD1099	CH_3	CH_3	SO_4CH_3	150	Temporary clearing at 50 mg./kg.	Delayed death at 50 mg./kg.	Delayed death at 50 mg./kg.	Delayed death at 50 mg./kg.
RD2216	CH_3	C_2H_5	$SO_4C_2H_5$	120	Delayed death at 32 mg./kg.	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.
RD2586	C_2H_5	CH_3	SO_4CH_3		,,	,,	,,	,,
RD2587	C_2H_5	C_2H_5	SO_4CH_3		,,	,,	,,	,,
RD1097	C_4H_9	CH_3	SO_4CH_3	50	Inactive at 20 mg./kg.	Inactive at 20 mg./kg.	Inactive at 20 mg./kg.	Inactive at 20 mg./kg.
RD1579		$(CH_2)_3N(C_2H_5)_2$	Cl	140	Delayed death at 40 mg./kg.	Inactive at 40 mg./kg.	Inactive at 40 mg./kg.	Inactive at 40 mg./kg.
RD2307		CH_3	Cl	*ca.* 500	,,	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.
RD2460		C_2H_5	Cl	>1600	,,	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.
RD2283		CH_3	SO_4CH_3	140	,,	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.	Delayed death at 32 mg./kg.
RD2389		C_2H_5	$SO_4C_2H_5$		Inactive at 40 mg./kg.	Inactive at 40 mg./kg.	Inactive at 32 mg./kg.	Inactive at 32 mg./kg.

quaternated compound, RD 2141, is extremely interesting, and further work on this type of compound is indicated.

Summary

1. Results of tests for trypanocidal activity on a number of phenanthridinium compounds are reported.

2. The effect of structure upon trypanocidal activity is discussed for each compound, one of which, 2: 7-diamino-9-phenyl-10-ethoxypropyl phenanthridinium methane sulphonate, has low acute toxicity for mice and high activity in trypanosome infections in mice.

I am grateful to Mrs. B. Goodliffe and Miss H. Riley for technical assistance, to Dr. T. I. Watkins, who has supplied all the compounds, and to Dr. M. R. Gurd for figures for toxicity of the compounds. The chemistry of the compounds will be reported elsewhere.

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Brit. J. Pharmacol. (1956), **11**, 339.

SOME ACTIONS OF CHLORPROMAZINE

BY

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From the Biological Research Laboratories, May and Baker Ltd., Dagenham

(RECEIVED JUNE 26, 1956)

Since the first account of the pharmacology of chlorpromazine by Courvoisier, Fournel, Ducrot, Kolsky, and Koetschet (1953), much work has been done in an attempt to elucidate the mode of action of this drug. Investigations have been made by Bradley and Hance (1955), Dasgupta and Werner (1954), Hiebel, Bonvallet, and Dell (1954), Holzbauer and Vogt (1954), Longo, Von Berger, and Bovet (1954), Cathala and Pocidalo (1952), Huidobro (1954), Kopera and Armitage (1954), Melville (1954), Cosnier and Drouin (1954), Salzman, Moran, and Brodie (1955), and others.

Chlorpromazine, like promethazine, is a phenothiazine derivative and possesses some of its actions to a greater degree, but it has negligible antihistamine properties. Perhaps the most outstanding property of chlorpromazine is its central depressant action, which is different from that of barbiturates, and is more marked than with other related phenothiazines. Its main peripheral action is an antagonism to adrenaline, and Kopera and Armitage (1954) showed that chlorpromazine also caused a paralysis of striated muscle. These authors compared the properties of chlorpromazine, promethazine, and pethidine, and found that all three substances showed the same types of pharmacological action, but chlorpromazine was the most active except in its anti-acetylcholine and antihistamine actions.

In the present investigation we have determined pA_2 values for the anti-acetylcholine and antihistamine properties of chlorpromazine. Its actions on striated muscle, on the heart for a quinidine-like effect, on body temperature, on insulin hypoglycaemia, and on liver glycogen, were also studied.

METHODS

Anti-acetylcholine and antihistamine actions were determined quantitatively on the guinea-pig ileum by measurement of pA_2 values (Schild, 1947). The antihistamine action was also estimated in guinea-pigs by the histamine aerosol method of Bovet and Walthert (1944). Atropine-like activity was determined on the pupil of the mouse by the method of Ing, Dawes, and Wajda (1945), the solutions of the compounds being injected intraperitoneally, or instilled into the eye.

The hypothermic action of chlorpromazine was compared with that of promethazine in rabbits; the drugs were injected subcutaneously and the rectal temperatures were recorded with thermocouples.

The quinidine-like activity of chlorpromazine and promethazine was determined by the method of Dawes (1946), after the drugs had been in contact with the auricles for 10 and 20 min.

Actions of chlorpromazine and promethazine on striated muscle were studied on the phrenic nerve-diaphragm preparation (Bülbring, 1946) of the young rabbit, the sciatic-gastrocnemius preparation of the cat (Bülbring and Burn, 1942) and of the guinea-pig (Hall and Parkes, 1953). The muscle was stimulated, either indirectly or directly, with square wave pulses at a rate of 8/min. In the cat and guinea-pig, injections were made intravenously, or into the tied contralateral iliac artery, close to the bifurcation of the aorta. Guinea-pigs were anaesthetized with urethane intraperitoneally, and cats with chloralose given intravenously.

The effect of chlorpromazine on the volume of the skinned hind limb in cats was measured by means of a plethysmograph and a piston volume recorder.

The ability of chlorpromazine to alter the response to insulin in animals was studied by two methods. In one method, convulsions with insulin were produced in albino mice deprived of food for 24 hr.; the mice were placed in glass jars in an air chamber thermostatically controlled at 36° C. The number of animals convulsing within 3 hr. and the deaths within 18 hr. were recorded. Injections were made intraperitoneally. In the other method, the blood-sugar of rabbits (deprived of food for 18 hr. before the experiment) was determined by the method described by Hagedorn and Jensen (1923). The injections were made subcutaneously and the blood-sugar was determined either hourly or in a pooled sample of blood collected at hourly intervals over a 5 hr. period. The action of chlorpromazine on liver glycogen, which was estimated by the method of Kahan (1953), was investigated in albino rats.

The drugs used were: chlorpromazine hydrochloride, promethazine hydrochloride, tubocurarine chloride, acetylcholine chloride, and histamine acid phosphate, and the doses or concentrations given are all expressed in terms of the salt. Quinidine was weighed as the base, dissolved in sufficient dilute hydrochloric acid, and the amounts are expressed in terms of the base.

Results

Anti-acetylcholine Action

On the guinea-pig ileum the mean pA_2 values for chlorpromazine were 6.07 (n=number of determinations=3) at 2 min. and 6.20 (n=2) at 14 min., with standard deviations of 0.22 and 0.04 respectively. Mean values for promethazine, obtained in these laboratories by Edge (1953), were 7.53 (n=2) and 7.81 (n=2), (S.D. 0.09 and 0.22 respectively). When a comparison was made with these results, chlorpromazine was 28 times less active than promethazine at 2 min. and 40 times less active at 14 min. Kopera and Armitage (1954) obtained a relative figure of approximately 8, but they left the antagonist in the bath for 1 min. before adding acetylcholine. Chlorpromazine was 1.3 times as potent at 14 min. as at 2 min. whereas promethazine was 1.9 times as potent at the longer period of contact.

The intraperitoneal injection of 5 mg./kg. of chlorpromazine had no effect on the pupil in mice observed for 1 hr. whereas this dose of promethazine caused a definite mydriasis in 15 min. However, the instillation of solutions of either chlorpromazine or promethazine into the eye produced a dilatation of the pupil. Ten mice at each concentration and three concentrations of each compound were used. Plotting log concentration against increase in pupil diameter gave dose-response curves which were similar in slope, and the relative potency of chlorpromazine to promethazine was 1/125. A concentration of 2.0% w/v chlorpromazine, for example, was found to be equivalent to a concentration of 0.016% w/v promethazine.

Antihistamine Action

On the guinea-pig ileum the mean pA_2 values for chlorpromazine were 7.67 (n=4) at 2 min. and 7.87 (n=3) at 14 min. (S.D. 0.34 and 0.10 respectively). Edge (1953) obtained corresponding values for promethazine of 8.01 (n=2) and 9:21 (n=2), (S.D. 0.06 and 0.05 respectively). A comparison of these two sets of figures showed that chlorpromazine was one half as potent as promethazine after 2 min. and 22 times less potent after 14 min. Kopera and Armitage found the relative potency to be one third after 1 min. contact. The activity of chlorpromazine at 14 min. was 1.6 times that at 2 min. whereas promethazine was 16.0 times as effective at 14 min. as at 2 min. (Edge, 1953). Thus the equilibrium condition, when the drug was exerting a maximum effect, was reached more quickly with chlorpromazine than it was with promethazine. At 2 min. contact the ratio of antihistamine to anti-acetylcholine activity was 39.5:1 for chlorpromazine and 3.0:1 for promethazine. Kopera and Armitage obtained similar values (33:1 and 4:1 respectively). Under equilibrium conditions, however, this difference between the two compounds was less marked; the ratios were 47.0:1 for chlorpromazine and 25.4:1 for promethazine.

The results obtained by using histamine aerosols (2% w/v histamine acid phosphate) in guinea-pigs (10 or 12 at each dose) are shown in Fig. 1. Chlorpromazine was about 40 times less active than promethazine, i.e. 20 mg./kg. of chlorpromazine was approximately equivalent in its effect to 0.5 mg./kg. of promethazine.

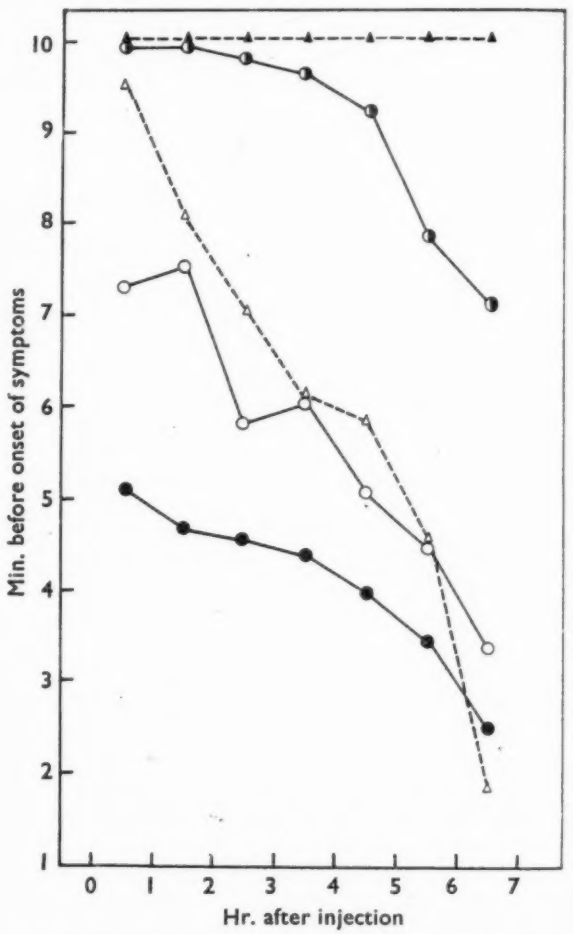

FIG. 1.—Antihistamine action by histamine aerosol method in guinea-pigs. Each point is the mean value of 10 or 12 animals. Ordinates: time (min.) before the animals collapsed due to respiratory distress. Abscissae: time (hr.) after subcutaneous injection of antihistamine compound. ●——●, chlorpromazine 10 mg./kg. ○——○, chlorpromazine 20 mg./kg. ◑——◑, chlorpromazine 40 mg./kg. △----△, promethazine 0.5 mg./kg. ▲----▲ promethazine 1.0 mg./kg.

Effect on Body Temperature

An assay was carried out in which three doses of both chlorpromazine and promethazine, and eight rabbits at each dose, were used.

The average maximum falls in temperature during the 4-hr. period following injection were plotted against the log dose (Fig. 2). Similar graphs were obtained when the mean sums of the eight deviations (at half-hourly intervals over four hours), from the temperature before injection, were plotted against the log dose. Relative potencies were determined from the antilog of the dose interval between the best fitting parallel straight lines, which were drawn by eye. Calculated from the maximum fall in temperature, the activity of chlorpromazine was 4.3 times that of promethazine, and the relative potency was 3.9 when calculated from the summed deviations in temperature.

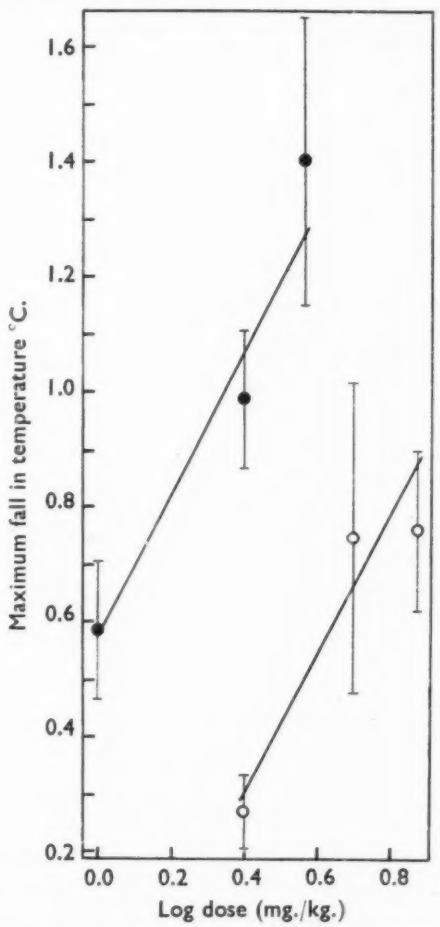

FIG. 2.—Hypothermic action in rabbits. Each point is the mean value of 8 animals. Subcutaneous injections. ●, chlorpromazine; ○, promethazine. Ordinates: maximum falls in temperature observed in 4 hr. Abscissae: log dose in mg./kg. Vertical bars are standard errors.

Quinidine-like Action

Both chlorpromazine and promethazine were as potent as quinidine in reducing the maximum rate of stimulation to which the auricles would respond without dropping beats. Each assay was carried out on a fresh preparation, in which two or three doses of quinidine, followed by only one dose of either chlorpromazine or promethazine, were used. The percentage reduction in the maximal rate of stimulation was plotted against the log dose. It was found that concentrations of about 5 μg./ml. produced similar effects. The mean relative potencies (quinidine=1.0) were 1.06 (n=number of assays=5) at 10 min., and 1.02 (n=3) at 20 min., for chlorpromazine, and 0.93 (n=2) at 10 min., and 1.0 (n=1) at 20 min., for promethazine, with standard errors of 0.38, 0.056, 0.075 respectively for the first three of these values. After these results were obtained it was noted that Schallek (1956) had also found chlorpromazine to have a quinidine-like action.

Effects on Striated Muscle

In conscious rabbits doses of 2–12 mg./kg. intravenously of chlorpromazine produced drowsiness, but not a head drop characteristic of neuromuscular blocking substances. A dose of 16 mg./kg. was fatal.

On the phrenic nerve-diaphragm preparation both chlorpromazine and promethazine had a dual action. First, there was an inhibition of neuromuscular transmission, shown by a failure of the muscle to contract when stimulated through the phrenic nerve; but there was little effect on the response to direct stimulation (Fig. 3a). This neuromuscular blocking effect, which was usually obtained with a concentration of about 14 μg./ml., was not reversed by neostigmine (0.4–1.2 μg./ml.). Secondly, there was an increase in resting tone and the heights of the contractions were reduced (Fig. 3b). This effect was observed after the concentration of drug in the bath was doubled, the response to indirect stimulation having been completely abolished at the lower concentration. A similar effect was obtained with the higher concentration, after abolition of the response to indirect stimulation with tubocurarine. It was noticed that after chlorpromazine had been washed out, following a period of contact of 5 min. or more, the responses to indirect and direct stimulation were reduced or even abolished, sometimes for only a few minutes and sometimes until the end of the experiment.

On the sciatic-gastrocnemius preparation of the cat, 3 mg./kg. of chlorpromazine or promethazine,

injected intra-arterially, first augmented and then decreased the contractions on indirect stimulation; these effects were similar to those obtained by Kopera and Armitage. However, intra-arterial injection of the same dose of chlorpromazine in cats previously curarized with tubocurarine produced inhibition of the contractions on direct stimulation, but there was no initial potentiation. In guinea-pigs, when chlorpromazine was given intra-arterially in the same dose, only inhibition of the contractions on indirect stimulation was obtained. On the other hand the effect of intravenous injection of chlorpromazine in cats was slight and often absent. In one cat about 30% inhibition of the contractions on indirect stimulation was obtained with a dose of 5 mg./kg.: in this cat there was a large fall in blood pressure. In four other cats no inhibition was observed after doses of 6 to 20 mg./kg. of chlorpromazine, when there was little effect on the blood pressure. The effect of chlorpromazine on the blood pressure was usually less after repeated doses. Intravenous injection in guinea-pigs of single or repeated doses of chlorpromazine up to 8 mg./kg. did not decrease the size of the muscle twitches on indirect stimulation.

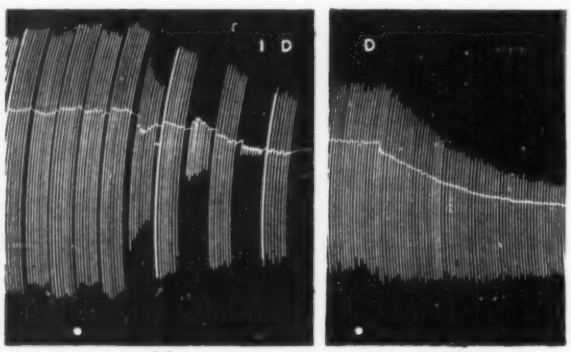

FIG. 3.—Rabbit phrenic nerve-diaphragm. Single supramaximal shocks (8 per min.). I, indirect stimulation (0.4 msec. duration). D, direct stimulation (15 msec. duration). Chlorpromazine, 2 mg. (bath capacity 140 ml.) at ○. Between (a) and (b), 30 min. interval; preparation not washed out. Time scale, 30 sec. Contraction downwards.

In a cat under chloralose the intra-arterial injection of 1 mg./kg. of chlorpromazine produced a transient increase in limb volume indicating a dilator effect in the muscles, since the skin was removed. It is noteworthy that a peripheral vasodilatation has been demonstrated in man (Foster, O'Mullane, Gaskell, and Churchill-Davidson, 1954). It is therefore unlikely that any local vasoconstriction could have accounted for the observed inhibitory effects on the neuromuscular system.

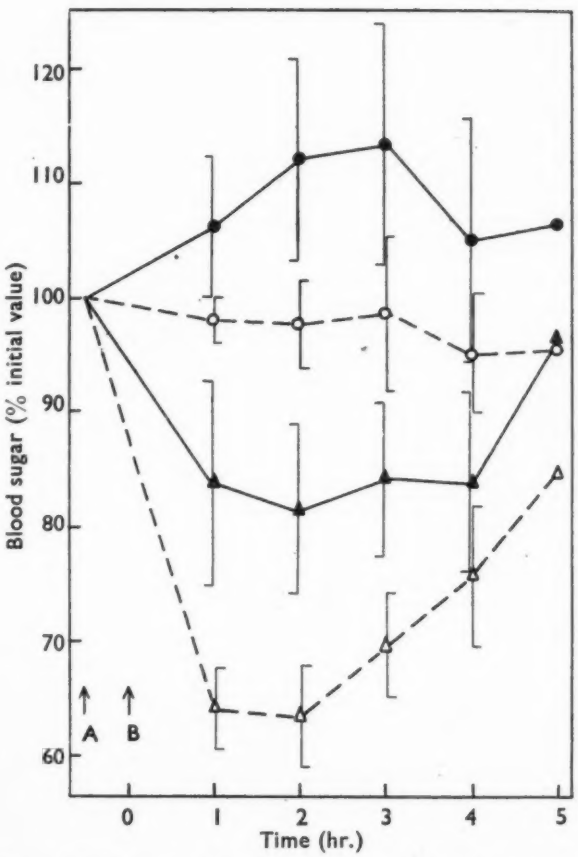

FIG. 4.—Blood-sugar values in rabbits, as % initial values. Each point is the mean of 8–11 animals. ●——●, chlorpromazine, 5 mg./kg. ○---○, controls. ▲——▲, chlorpromazine, 5 mg./kg.+insulin, 0.125 u./kg. △---△, insulin, 0.125 u./kg. Subcutaneous injections. Chlorpromazine injected at A and insulin at B. Ordinates: blood-sugar as % of initial value. Abscissae: time (hr.). Vertical bars are standard errors.

Effects on Blood-sugar and on Liver Glycogen

When chlorpromazine was given subcutaneously to rabbits in a dose of 5 mg./kg., there was a very small hyperglycaemia. More interest was attached to the effect chlorpromazine might have in modifying the effects of insulin on the blood-sugar in rabbits, and on the convulsive effect of insulin in mice.

Effect on Insulin Hypoglycaemia.—Chlorpromazine, given subcutaneously to rabbits in a dose of 5 mg./kg., half an hour before insulin, reduced the hypoglycaemia (Fig. 4). The mean blood-sugar values were not significantly different at any of the hourly points. However, the mean value over the 5 hr. period, for rabbits given chlorpromazine and insulin, was significantly higher than for animals given insulin alone ($P < 0.001$).

The results of an 8 ×8 Latin square experiment on eight rabbits are shown in Table I. The treatments were two doses of insulin, three doses of

TABLE I

EFFECT OF CHLORPROMAZINE ON MEAN BLOOD-SUGAR VALUES IN RABBITS

Blood samples were pooled over 5 hr. and the values expressed as % of the initial value. (Subcutaneous injections)

Insulin (u./kg.)	Chlorpromazine (mg./kg.)	No. of Rabbits	Mean	S.E.
0·125	Nil	8	78·5	6·0
0·0625	,,	7*	84·4	5·9
0·125	10·0	8	108·5	13·1
0·125	5·0	8	103·5	9·8
0·125	2·5	7*	85·3	11·8
Nil	10·0	7*	141·8	21·9
,,	5·0	8	144·1	27·8
,,	2·5	7*	112·1	2·1

* Because of the death of 1 rabbit before completion of the experiment, there were only 7 observations in each of these groups.

chlorpromazine, and three doses of chlorpromazine in conjunction with insulin. All injections were given subcutaneously. Doses of 5 and 10 mg./kg. of chlorpromazine abolished the hypoglycaemia due to insulin, and all three doses caused a significant hyperglycaemia, more marked than in the previous experiment. The blood-sugar values of the five animals which received insulin (i.e. three given insulin and chlorpromazine and two given insulin alone) were summed for each of the eight days of the experiment. The means of the figures so obtained, plotted against the day of the experiment, gave a straight line with a slope of 5.46 which differed significantly from zero ($P<0.01$). This indicated either that chlorpromazine had a cumulative effect, or that there was a decreased sensitivity to insulin.

TABLE II

EFFECT OF CHLORPROMAZINE ON INSULIN CONVULSIONS AND DEATHS IN MICE

(Intraperitoneal injections)

Insulin (u./kg.)	Chlorpromazine (mg./kg.)	No. of Mice	Convulsed		Died	
			No.	%	No.	%
1·0	Nil	48	33	69	27	56
0·8	,,	48	26	54	9	19
0·64	,,	48	19	39	9	19
1·0	10	24	10	42	8	33
1·0	7·1	48	16	33	6	12
1·0	5·0	48	19	40	9	19
1·0	3·6	24	7	29	6	25

Effect on Insulin Convulsions.—Chlorpromazine reduced the number of mice convulsing and also the number of deaths after insulin (Table II). No relationship between the dose of chlorpromazine and the reduction of convulsions was obtained. However, there was a maximum protection against the lethal effects of insulin with a dose of 7.1 mg./kg. of chlorpromazine. The small protection given by 10.0 mg./kg. might have been due to a toxic action of chlorpromazine itself. χ^2 tests showed that the reduction in the number of mice convulsing after chlorpromazine was significant; the results on all groups which received chlorpromazine were summed and compared with a control group ($P<0.001$). The number of animals which died was also significantly reduced after chlorpromazine ($P<0.001$).

Effect on Liver Glycogen.—In order to determine whether the effects of chlorpromazine on blood-sugar were due to a depletion of the glycogen stores in the liver, the following experiments were carried out in albino rats. So that any inhibitory effect might be more readily detected, the stores of glycogen, in fasted rats, were previously raised either by the administration of glucose or by recent feeding. In some experiments the rats were deprived of food for 24 hr. and then 0.5 g. of glucose was given by mouth to each rat. This amount was found to be a convenient quantity to administer in order to produce a temporary rise in the liver glycogen (Barnes, 1953, used a similar amount in studying the effect of dinitro-orthocresol on glycogen deposition in the liver). The liver glycogens after glucose, where each figure was the mean result of determinations on 6 rats weighing 150–300 g., were:

Hours	0	1	2	4	6	8	24
Liver glycogen %	0·35	0·52	0·62	0·54	0·52	0·38	0·50

0·5 g. glucose, orally

After 0.5 g. glucose there was a moderate rise in liver glycogen which was maintained between 1 and 6 hr. Consequently, to test the effect of chlorpromazine it was injected subcutaneously either 3 or 4½ hr. after glucose, and the first liver glycogen estimations were made between 1 and 2 hr. later. In other experiments, instead of giving glucose, each rat had 10 g. of a stock diet the evening before an experiment. These results are shown in Table III. The effects obtained with chlorpromazine in these experiments were not consistent. Although a temporary depletion of liver glycogen was obtained in experiment 1, where the difference at 1½ hr. was significant ($P<0.05$), in the other experiments there was no marked depletion, and in fact in experiments 3, 4, and 5 there was a slight increase.

DISCUSSION

In these experiments some of the properties of chlorpromazine have been determined quantitatively. It was found, from pA_2 determinations against acetylcholine and histamine on the isolated guinea-pig ileum, that equilibrium conditions were not obtained with chlorpromazine at 2 min. period of contact. Edge (1953) obtained similar results for

TABLE III

EFFECT OF CHLORPROMAZINE ON LIVER GLYCOGEN

Liver glycogens (%), with standard errors; 6 or 8 rats were used for each determination; Expts. 1–3 after glucose, 4 and 5 after feeding a stock diet. In Expts. 1 and 2 chlorpromazine was given subcutaneously 4½ hr. after glucose, and 3 hr. after glucose in Expt. 3

Body Wt. (g.)	Expt. No.		mg./kg.	Hr. after Chlorpromazine						
				1	1½	2	3	3½	4	20
150–300	1	Chlorpromazine	10	—	0·26±0·03	—	—	0·37±0·03	—	0·44±0·03
		Controls		—	0·52±0·03	—	—	0·32±0·03	—	0·47±0·03
70–110	2	Chlorpromazine	10	—	0·51±0·04	—	—	—	—	—
		Controls		—	0·66±0·04	—	—	—	—	—
150–300	3	Chlorpromazine	10	0·38±0·03	—	—	0·35±0·03	—	—	0·46±0·03
		Controls		0·36±0·03	—	—	0·27±0·03	—	—	0·32±0·03
70–110	4	Chlorpromazine	10	—	—	5·24±0·71	—	—	3·69±0·86	—
		Controls		—	—	3·77±0·71	—	—	4·10±0·86	—
70–110	5	Chlorpromazine	20	—	4·02±0·49	—	—	—	—	—
		Controls		—	3·47±0·49	—	—	—	—	—

promethazine. It was found that pA_2 values determined under equilibrium conditions (i.e. at 14 min. period of contact) gave closer agreement with other results *in vivo*, obtained by the histamine aerosol and mouse pupil methods.

In rabbits chlorpromazine was only about four times as potent as promethazine in lowering body temperature, in contrast to a twenty-five times difference in mice (Courvoisier *et al.*, 1953). This difference between the relative potencies might be related to the part which surface cooling plays in the regulation of body temperature in the two species. That surface cooling is a factor in the hypothermic action is supported by the observations of Giaja and Markovic-Giaja (1954) that the degree of hypothermia after chlorpromazine in rats varied with the environmental temperature, and of Halpern and Liakopoulos (1954), who showed that the hypothermic action of chlorpromazine, but not of promethazine, was enhanced by a low environmental temperature. Chlorpromazine possibly affects surface cooling by means of its peripheral vasodilator action (Foster *et al.*, 1954).

It has been suggested that the fall in body temperature after chlorpromazine is partly due to a depressant effect on striated muscle (Kopera and Armitage, 1954). Though this might be true for some species, it could not be the explanation in guinea-pigs, at least where intravenous injection of large doses of the drug had no depressant effect on the gastrocnemius muscle, although the drug does produce a fall in body temperature (Halpern and Liakopoulos, 1954).

It was concluded that chlorpromazine depressed the activity of striated muscle only when the drug reached a very high concentration at the site of action, since inhibitory effects on the sciatic-gastrocnemius preparation were only consistently obtained after intra-arterial injection; intravenous injection in cats gave inconsistent results. Both chlorpromazine and promethazine had a dual action on the phrenic nerve-diaphragm preparation of the rabbit, producing a neuromuscular block followed by a direct effect on the muscle. The neuromuscular block was not due to competitive inhibition, since the effect was not reversed by neostigmine. We noted the similarity between these effects and those described by Hajdu and McDowall (1949), who attributed the effects, on neuromuscular transmission, of phloridzin or lack of glucose to a failure of acetylcholine synthesis at the nerve endings.

Burn (1954) has pointed out that the quinidine-like action of chlorpromazine could be expected from a knowledge of its other properties. It was not surprising, therefore, that the drug resembled quinidine in its action on the electrically-driven isolated auricles of the rabbit, besides inhibiting the fibrillatory action of aconitine and other agents (Courvoisier *et al.*, 1953) and of pressor amines (Melville, 1954).

Chlorpromazine reduced the hypoglycaemia and convulsions due to insulin in experimental animals. Norman and Hiestand (1955) have also shown, in mice, that convulsions due to insulin were reduced after chlorpromazine. Since chlorpromazine and insulin therapy are sometimes used concurrently it is of interest that Lancaster and Jones (1954) were not able to demonstrate a significant effect upon hypoglycaemia due to insulin in man, but they used much smaller doses of insulin than would normally be employed for the treatment of psychiatric cases. However, Merivale and Hunter (1954) and Charatan and Bartlett (1955) showed that after chlorpromazine glucose-tolerance curves were abnormal, indicating

that chlorpromazine might have some action on carbohydrate metabolism in man.

The transient depletion, after chlorpromazine, of the liver glycogen in one experiment when glucose was given 4½ hr. previously was unlikely to be due to an effect on absorption, since in fed rats, and in rats which had been given glucose only 3 hr. previously, there was no depletion. It may have been that the alimentary hyperglycaemia, due to the absorption of carbohydrate from the intestine in the latter experiments, in some way prevented the slight depletion of glycogen stores. In any case, in view of its transient and inconsistent nature this effect cannot be considered very important. However, Cosnier and Drouin (1954) noted a depletion of the liver glycogen and an increase in the fat content of the liver cells, but these workers used a histological technique.

Summary

1. Under equilibrium conditions (14 min. contact) on the isolated guinea-pig ileum, chlorpromazine was 40 times less potent than promethazine against acetylcholine and 22 times less potent against histamine. It was 125 times less active than promethazine on the mouse pupil and 40 times less active by the histamine aerosol method.

2. Chlorpromazine was four times as active as promethazine in lowering the body temperature in rabbits.

3. The quinidine-like action of chlorpromazine on rabbit auricles was similar to that of promethazine, and both of the phenothiazine compounds were as active as quinidine itself.

4. Chlorpromazine had an inhibitory action on skeletal muscle in cats and guinea-pigs only when injected intra-arterially; intravenous injection in cats gave variable results and was without effect in guinea-pigs. On the isolated phrenic nerve-diaphragm of the rabbit there was an inhibition of neuromuscular transmission and little effect on the maximum tension on direct stimulation, and, at a higher concentration, a direct effect on the muscle.

5. The incidence of convulsions due to insulin in mice was reduced by chlorpromazine. It produced a slight hyperglycaemia and reduced a hypoglycaemia due to insulin in rabbits. Chlorpromazine had little effect on the deposition of liver glycogen in rats.

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THE EFFECT ON RESPIRATION OF INFUSIONS OF ADRENALINE AND NORADRENALINE INTO THE CAROTID AND VERTEBRAL ARTERIES IN MAN

BY

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Intravenous injections of adrenaline and noradrenaline in man cause a striking increase in the rate and depth of respiration. This is associated with a fall in the alveolar CO_2 content and an increase in the respiratory minute volume. The hyperpnoea is abrupt in onset and does not appear to be accounted for by a general increase in metabolic rate (Whelan and Young, 1953).

The present experiments were carried out in an attempt to determine if the stimulant effect of adrenaline and noradrenaline on respiration in man is the result of a direct action on the respiratory or other centre in the brain.

METHODS

The subjects were patients on whom cerebral angiography was being carried out for diagnostic purposes. Following the first injection of contrast medium, an interval elapsed, while the films were developed and studied, before further injections were made. Advantage was taken of this interval to infuse small doses of adrenaline or noradrenaline and record their effects on respiration.

The patients lay supine with the head extended. The respiratory movements were recorded by means of two stethographs, one around the chest and the other around the abdomen (Shepherd, 1951 ; Dornhorst and Leathart, 1952). These were connected to a volume recorder which traced the movements with an ink writer on a kymograph drum.

Direct Infusion into the Common Carotid Artery.—Under local anaesthesia with 10–20 ml. of 1% (w/v) procaine, a needle, 9.5 cm. long and 2 mm. in external diameter with a short bevel, was inserted into the common carotid artery. The needle was connected to a mechanically-driven syringe by a length of polythene tubing. An infusion of 0.9% saline was maintained throughout at a rate of 4 ml./min. and was interrupted when required either for injection of contrast medium or for the infusion of adrenaline or noradrenaline solutions.

The contrast medium used was 35% (w/v) diodone given in quantities of 12 ml. for carotid angiograms and 14 ml. for vertebral angiograms. In one patient sodium acetrizoate (30% w/v) was used for the vertebral angiograms. The drug solutions were made up in sterile saline (containing 0.001% w/v ascorbic acid) from (—)-adrenaline tartrate (B.D.H. 1:1000) and (—)-noradrenaline tartrate (Levophed, Bayer) so that the minute dose was contained in 4 ml. The doses are expressed as weights of the salts. Adrenaline has been shown to remain stable in ascorbic acid saline for many hours (Gaddum, Peart, and Vogt, 1949).

Indirect Infusions into the Vertebral Artery.—In those patients on whom right-sided angiograms were being performed, once the direct common carotid infusion had been established the infusion was diverted into the vertebral artery as follows. A sphygmomanometer cuff around the right upper arm close to the axilla was inflated to 240 mm. Hg and pressure with the thumb was applied over the common carotid artery just above the position of the needle. The abolition of pulsations in the superficial temporal artery indicated successful occlusion of the common carotid. Injected fluid was thus diverted into the vertebral artery via the subclavian artery. This technique was similar to that described by Engeset (1948) and Ecker (1951), and resulted in varying amounts of contrast medium passing into the vertebral circulation.

Direct Infusion into the Vertebral Artery.—Vertebral angiograms and infusions were carried out through a needle inserted directly into the vertebral artery, the needle being passed between the transverse processes of the fourth and fifth cervical vertebrae (Fig. 1), after the manner described by Sugar, Holden, and Powell (1949) and Lindgren (1950). Five patients were under light general anaesthesia (N_2O and O_2) but were breathing naturally and did not require assisted respiration at any time. In one patient the procedure was carried out under local anaesthesia with 1% (w/v) procaine.

RESULTS

Intra-carotid Infusions.—In four patients adrenaline was infused at rates ranging from 0.01 to

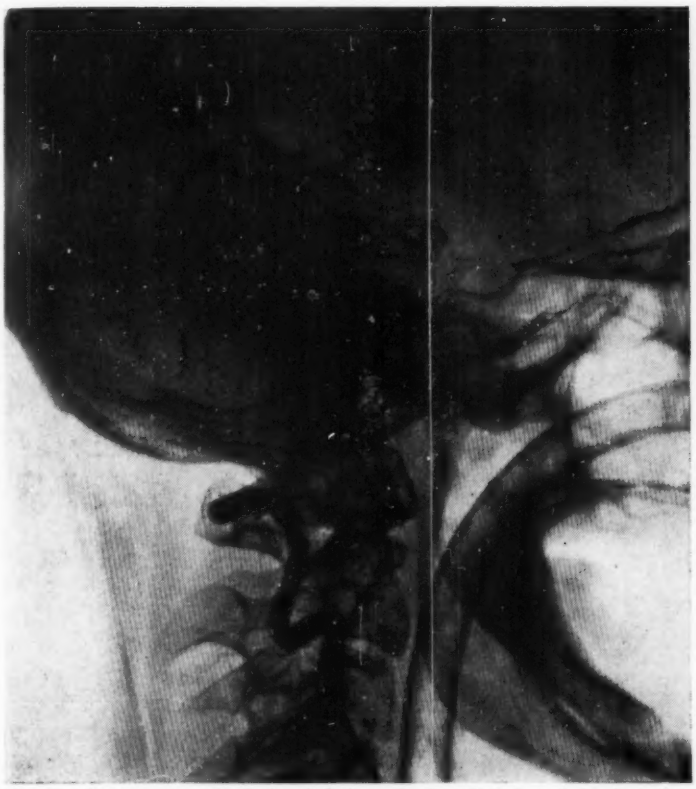

FIG. 1.—Angiogram made during infusion of adrenaline 1 μg./min. into the right vertebral artery in patient S. T. 14 ml. 35% diodone was injected.

5 μg./min. into the left common carotid artery, and in five other patients adrenaline or noradrenaline or both were given at rates of 1–2 μg./min. into the right common carotid artery. The pattern of respiration was not altered by any of these infusions. In two of the patients an infusion of 10 μg./min. was given ; this produced not only an increase in the depth and rate of respiration, but was accompanied by pallor, an increased pulse rate and palpitation, and was probably due to the drug passing into the general circulation, since this rate of infusion produces the same general effects when given intravenously (Barcroft and Swan, 1953).

Indirect Vertebral Infusions.—In preliminary experiments, which have been reported elsewhere (Duff, Shepherd, and Whelan, 1954), it was found that the diversion of adrenaline infusions from the common carotid into the vertebral artery by the technique described above resulted in stimulation of respiration. It has subsequently been found, however, that pressure alone over the common carotid artery caused, in four out of five patients, an increase in rate and depth of respiration. In the fifth patient neither the pressure alone nor pressure during adrenaline infusion had any effect on the respiratory pattern. Thus the effects of adrenaline infusions diverted into the vertebral artery by carotid occlusion could not be differentiated from the effects of carotid occlusion alone. This method of infusing the vertebral artery was, therefore, abandoned in favour of direct puncture of the vertebral artery itself.

Direct Vertebral Infusions.—Six patients requiring vertebral angiography were studied, and in no case did adrenaline or noradrenaline have any effect on the respiration when infused directly into the vertebral artery in doses of 1–2 μg./min. Fig. 2 (S.T.) shows the records obtained in the patient whose angiogram taken during the adrenaline infusion is illustrated in Fig. 1. In Fig. 2 (J. M.) the contrast medium sodium acetrizoate was used to obtain the angiogram, and injection of this had a transient inhibitory effect on the respiration. In Fig. 2 (R. G.) the respiratory record obtained during infusion of adrenaline directly into the vertebral artery at 2 μg./min. is compared with that during intravenous infusion at 10 μg./min. five minutes later. The intravenous infusion of adrenaline caused an increase in the rate and depth of respiration similar to that seen in conscious subjects, but infusion into the vertebral artery had no such effect.

In a seventh patient an attempt was made to eliminate the likelihood of streaming of the infused drug solution by giving as a single sudden injection 10 ml. of a solution of adrenaline in a concentration of 10 μg./litre into the vertebral and the internal carotid arteries. Before each injection 8 or 10 ml. of contrast material was injected in 3 seconds and shown by *x*-ray to fill the vessel and its branches. Adrenaline solution was then injected in the same way and in the same time, and presumably also filled the vessel and its branches. Ten ml. of saline alone was also injected as a control. Neither the adrenaline solution nor the saline caused any alteration in the respiration, though the major branches at least of the injected vessel were filled with the adrenaline solution, of a concentration greater than that calculated as likely to arrive in the arterial blood during an intravenous infusion at 10–20 μg./min.

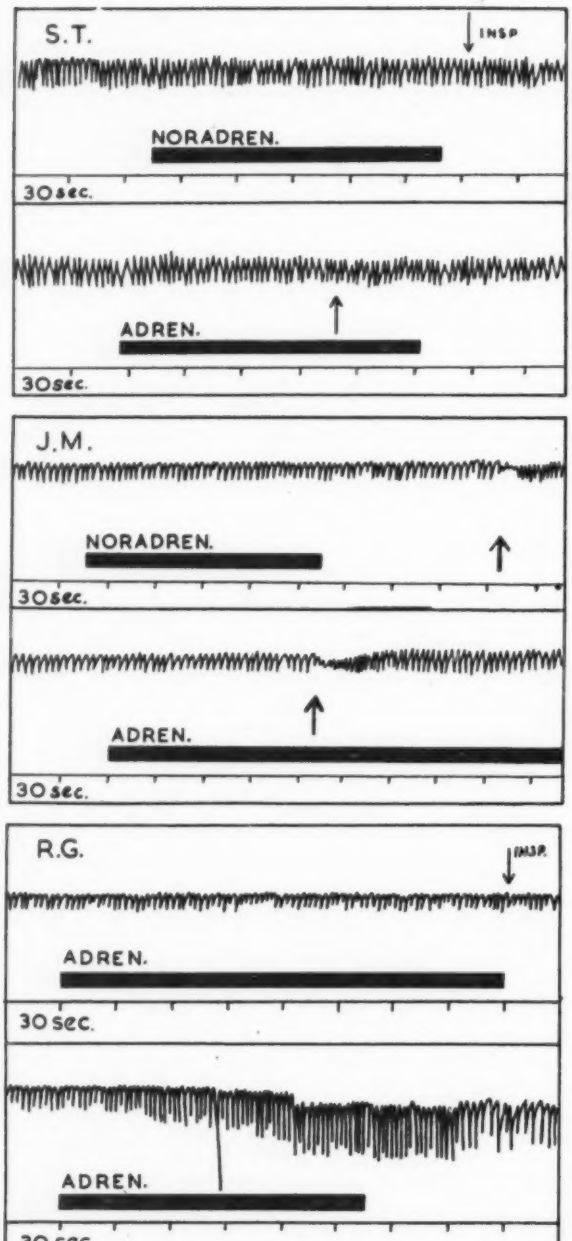

FIG. 2.—S. T.: Records of respiratory movements (inspiration downwards) during infusions of adrenaline and noradrenaline into the vertebral artery under light general anaesthesia. The black rectangles represent the duration of infusions and the arrow beneath the respiratory record indicates the time of injection of contrast (diodone). J. M.: Lightly anaesthetized patient. Contrast injected at arrows was sodium acetrizoate. R. G.: Comparison of the effect of intravertebral infusion of adrenaline (upper frame) and intravenous infusion (lower frame) 5 min. later in the same patient.

DISCUSSION

Stimulation of the respiration in man by the intravenous infusion of adrenaline has been noted by many workers (Tompkins, Sturgis, and Wearn, 1919; Lyman, Nicholls, and McCann, 1923; Cori and Buchwald, 1930; and Courtice, Douglas, and Priestley, 1939), but no explanation of this effect has been put forward.

Reale, Kappert, Skoglund, and Sutton (1950) reported an increase in the rate and depth of respiration with noradrenaline and attributed the increased amplitude to a compensatory reaction to decreased lung volume.

In a comparison of the effects of adrenaline and noradrenaline on respiration, Whelan and Young (1953) found that both amines had essentially the same stimulant effect on ventilation, but that the effect on oxygen consumption was very different, adrenaline causing an increase of about 32% whereas noradrenaline had no consistent effect. This difference in action of the two amines, associated with the abrupt onset of hyperpnoea within a minute or less of starting the infusion, made it unlikely that the increase in respiration was caused by a general increase in metabolism.

It is clear from the present results that adrenaline and noradrenaline infusions of 1–2 μg./min. into the common carotid and vertebral arteries in man have no effect on respiration. A stimulation of respiration is, however, brought about by intravenous infusion of 10–20 μg./min. of adrenaline. With a cardiac output of five litres/min. this represents an arterial blood concentration of 2–4 μg./litre. An infusion of 2 μg./min. into the vertebral artery represents a blood concentration of about 10 μg./litre if one assumes a total cerebral blood flow of 800 ml./min. (Kety and Schmidt, 1948) and the flow through one vertebral artery to be one quarter of this. The doses infused (1–2 μg./min.) were thus in excess of the amount calculated to arrive via the vertebral or internal carotid artery during intravenous infusions.

The injection of contrast medium during the infusion of the amines served to demonstrate that the needle was in fact inserted into the carotid or vertebral artery. It did not necessarily follow that the branches of the vessel outlined by the contrast were also perfused by the drug solution. This may well have had a different distribution on account of the differing dynamic effects of the two forms of introduction of the substances into the vessels.

In the present discussion the assumption is made that there is complete mixing of the drug infusion with the blood stream. It is, however, theoretically possible that laminar flow in the vessels might

have resulted in the occasional failure of adrenaline or noradrenaline to enter the branch supplying the respiratory centre or carotid body. While it is not possible to say whether such an occurrence accounts for the negative results of the present study, it is felt that it is unlikely to have taken place on every occasion on which the drugs were infused, particularly since the position of the needle in the artery varied slightly from experiment to experiment and even during the course of many of the experiments. Furthermore, the effect of single sudden injections of adrenaline solution in one patient was no different from that of the slower infusions.

It is therefore reasonable to conclude that the stimulant effects of intravenous infusions of adrenaline or noradrenaline cannot be attributed to a direct effect on a cerebral centre, and an indirect mechanism must be sought. Possibilities to be considered are stimulation of chemoreceptors in the carotid body or elsewhere, changes in blood pressure brought about by these amines, or a secondary release of a hormone or metabolite, or even a breakdown product of the amine itself. A reflex effect from the stimulation of the carotid body might have been expected during the intracarotid infusions. The rise in systolic arterial pressure during intravenous adrenaline infusions at 10 μg./min. is only 5–10 mm. Hg (Barcroft and Swan, 1953) and the mean pressure either falls or shows no change. Noradrenaline increases both the systolic and diastolic pressures by about 5–10 mm. Hg. It seems unlikely that these alterations in pressure could be responsible for the marked respiratory changes seen to a similar degree with both amines.

During the intravenous infusion of adrenaline there is an increase in the concentration of lactic acid in the peripheral venous blood (Bearn, Billing, and Sherlock, 1951 ; Barcroft and Cobbold, 1956). While the increase in lactate might account for the respiratory stimulant effect of adrenaline it cannot account for the effect of noradrenaline since there is no significant increase in the peripheral venous lactic acid concentration during infusion of the latter amine (Bearn, Billing, and Sherlock, 1951).

Bradley, Gaskell, Holland, Lee, and Young (1954) found no lowering of the arterial plasma *p*H during intravenous infusions of adrenaline in the human subject; on the contrary, an increase accompanied the hyperpnoea. They considered that the stimulant effect of adrenaline on respiration could not be the result of release of acid products of metabolism. From their studies on anaesthetized animals these workers concluded that the stimulation of respiration by adrenaline was not a direct action on the respiratory centre but due to a reflex from the thoracic region (Bradley *et al.*, 1953).

The results of the present experiments demonstrate that in the conscious or lightly anaesthetized human subject the stimulant action of adrenaline and noradrenaline on the respiration is not due to a direct action on the respiratory or other centre in the brain receiving its blood supply from the carotid or vertebral arteries.

Summary

1. Infusions of adrenaline and noradrenaline into the common carotid and the vertebral arteries during cerebral angiography in man did not cause any alteration in the respiratory pattern.

2. Angiograms taken during the drug infusions outlined the arteries and their branches.

3. It is concluded that the respiratory stimulant effect of adrenaline and noradrenaline when given intravenously cannot be a result of direct stimulation of a controlling centre receiving its blood supply from the carotid or vertebral vessels.

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